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The Interplay between Pain/Stress, Inflammation and Microbiota in Human Health

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Pain/stress, microbiome, and immune responses have emerged as key elements regulating human's health and disease. However, the mechanisms of their interplay with each other remain unclear. This manuscript style dissertation focused on the understanding of how pain/stress and microbiota influence the immune system and regulate the immune response. First, a nationwide online survey was conducted to develop an objective instrument to quantitatively measure cumulative pain/stress among NICU infants. Secondly, blood microbiome was investigated to examine the association between microbial translocation and plasma anti-CD4 autoantibodies in HIV+ subjects under long-term viral suppressive ART treatment. We found that plasma anti-CD4 IgG level was associated with elevated microbial translocation, reduced microbial diversity and distinct microbial composition in HIV+ patients, suggesting that systemic microbial translocation and microbiome may associate with anti-CD4 autoantibody production in ART-treated HIV disease. Lastly, a secondary data analysis was conducted to examine the relationship between cumulative pain/stress and Fecal calprotectin (FCP) level, a measurement of gut inflammatory responses, in preterm infants. We show that chronic (prolonged) pain/stress derived from daily medical procedures in the neonatal intensive care unit is positively associated with FCP levels. The study provides, to the best of our knowledge, the first insight of the modulation of prolonged pain/stress in the early programming of the gut immune system in preterm infants. These results suggest pain/stress and microbiome are essential factors in regulating immune responses. Understanding the mechanism will help with the development of an individualized intervention to restore immune function and improve health.

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CHAPTER ONE

There is a growing emphasis on the microbiota and its impact on human's health and disease especially the neurobehavioral responses and immune status. The interplay between the compositional and functional complexity of human microbiota and neuro-immune systems is often described as the brain-gut signaling system. Emergent research has shown that microbiota is crucial in regulating immune-inflammatory responses and inducing stress-related changes in behavioral and physiological responses (Bailey, 2014; Bharwani et al., 2016). Multiple factors have been identified influences the microbiota-gut-brain axis, including diet, physical activity and medication. Evidence has supported that brain-gut microbiome axis is a promising mechanism linking stress, inflammation, and microbiota in human's health (Cong, Xu, Romisher, et al., 2016). This manuscript style dissertation focused on exploring the relationship between pain/stress, microbiome and host immunity. The aim is to expand our understanding of early life stress and microbiome patterns and their roles in the modification of the immune-inflammatory processes.

1.1 Brain-gut microbiota axis

The concept of the brain-gut-microbiota axis is used to describe the bidirectional interaction between the central nervous system and peripheral gastrointestinal (GI) functions. This bidirectional communication network enables top-down signaling from the brain to influence the motor, sensory and secretory modalities of the GI tract. And conversely, the bottom-up signaling from the gut affects brain function, especially hypothalamus and amygdala that are devoted to emotion and stress (Carabotti, Scirocco, Maselli, & Severi, 2015; Galley et al., 2014).

The intestinal microbiota is involved in modulating peripheral GI function in several different ways, including affecting intestinal permeability, altering mucosal immune function,

interfering enteric reflex and entero-endocrine signaling (Mayer et al., 2015). Microbiota and its metabolites function somewhat like an endocrine organ and have been found associated with inflammatory and autoimmune conditions (Shen & Wong, 2016). The composition and function of microbiota has profound effects in the development and maturation of immune cells including natural killer T cells and regulatory T cells (Hrncir, Stepankova, Kozakova, Hudcovic, & Tlaskalova-Hogenova, 2008; Wei et al., 2010). Dysbiosis also damage the intestinal chemical and physical barriers, and impact the function of the immune factors such as secreted soluble immunoglobulin A and antimicrobial peptides (Sommer & Backhed, 2013). Evidence also suggests that changes in the composition of the gut or dysregulations of the microbial community are correlated with anatomy changes in central nerves system and altered brain functions such as emotional behaviors and social interactions (Mayer et al., 2015).

Several body systems are involved in the bidirectional brain-gut communication network, including the central nervous system (CNS), the autonomic nervous system (ANS), the enteric nervous system (ENS) and the hypothalamic pituitary adrenal axis (HPA) (Cong, Henderson, Graf, & McGrath, 2015). The ANS includes both the sympathetic nervous system and the parasympathetic nervous system. It regulates gut function by transmitting the afferent signals from the GI lumen through enteric, spinal and vagal pathways to the CNS, and efferent signals from CNS to the intestinal wall, targeting on the ENS, muscle layers and gut mucosa. Both branches of the ANS affect gut functions including motility, secretion, immune response and permeability of the intestine. The ANS also modulates the response of the gut immune cells including macrophages and mast cells to luminal bacteria with antimicrobial peptides. Additionally, stressful stimuli activate the HPA axis, leading to changes in the intestinal barrier

and gut microbiota, which increases gut permeability, allowing for bacterial invasion across epithelial barrier, and ultimately leading to systemic effects (Cong et al., 2015).

1.2 Pain and Stress

The International Association for the Study of Pain has defined pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage” ("Pain terms: a list with definitions and notes on usage. Recommended by the IASP Subcommittee on Taxonomy," 1979). Stress is defined as “a physical, chemical, or emotional factor that causes bodily or mental tension and may be a factor in disease causation” (Merriam Webster's Collegiate Dictionary, 1994).

Studies have shown that pain and stress impact the gut microbial community, and modulate the bacterial composition and function (Bailey, 2012). Physical and emotional stress, activates HPA and ANS to the intestine and reduces vagal output to the stomach, which is likely to change the composition and organizational structure of the gut microbiota, increase permeability of the intestinal epithelium, activate immune response in GI system and result in alterations of the GI function (Carabotti et al., 2015; Konturek, Brzozowski, & Konturek, 2011; O'Mahony, Hyland, Dinan, & Cryan, 2011; O'Mahony et al., 2009).

Acute procedural pain/stress, even during a very short period, can activate fight-or-flight responses, and prepare the organisms for possible infection and injury by stimulating the immune system and up-regulating both innate and adaptive immune responses (Dhabhar, 2014). Similar to a single acute pain/stress stimulus, prolonged chronic pain/stress has also been found associated with suppression of both cellular and humoral immunity in human adults (Segerstrom & Miller, 2004). Persistent pain/stress dysregulates innate and adaptive immune responses by stimulating pro-inflammatory cytokine, inducing chronic inflammation, and suppressing host

immune function (Chapman, Tuckett, & Song, 2008; Dhabhar, 2014). Cumulative pain/stress is more detrimental to health outcomes in neonatal infants where neuro-immuno-wiring and gut microbiome patterns are under formulation and thus, more easily altered. Persistent pain and stress during infancy can cause neuro-behavioral deficits and these alterations may last till adulthood (Beauchamp et al., 2008; Grunau, Holsti, & Peters, 2006; Grunau et al., 2009; Hermann, Hohmeister, Demirakca, Zohsel, & Flor, 2006). Exposure to prolonged painful/stressful events in the NICU has been found to be associated with reprogramming of the immune system, including depressed cortisol responses in preterm infants during NICU stay, and elevated cortisol level at 8 and 18 months corrected age (Grunau et al., 2007; Grunau et al., 2005).

Despite the fact that strong evidence shows uncontrolled pain/stress has destructive consequences, particularly in preterm infants, few instruments are available to assess the cumulative and persistent pain/stress. Majority of the existent tools are used to assess one-time acute pain/stress, which fails to provide a picture of long-term stress levels, especially in newborns. Few instruments have been developed to measure cumulative pain/stress, e.g., Neonatal Infant Stressor Scale (NISS); developed in Australia (Newnham, Inder, & Milgrom, 2009), and the generalizability of these tools to the population in the U.S needs to be tested.

1.3 Human Microbiota

Microbiota refers to the ecological community of commensal, symbiotic, and pathogenic microorganisms that reside in human's body (Group et al., 2009; Lederberg & McCray, 2001). Approximately $3.8 \cdot 10^{13}$ microorganisms live in human beings and play an essential role in human health and disease (Davis & Bajaj, 2017; Halfvarson et al., 2017; Maruvada, Leone, Kaplan, & Chang, 2017; Sender, Fuchs, & Milo, 2016; Tremlett, Bauer, Appel-Cresswell,

Finlay, & Waubant, 2017; Wu & Lewis, 2013; Young, 2017; Zhang & Zhao, 2016). Human microbiota has been classified into five types, including bacteria, archaea, protists, fungi, and virus. Human microbiota inhabit many human organs and tissue, most particularly the GI system (Sender et al., 2016). Microbial community can be considered as an essential organ and benefits human in many different ways, including providing essential nutrients such as hormones and vitamins to maintain proper function of the human body, metabolizing indigestible compounds and fermenting indigestible dietary fiber into short-chain fatty acids (SCFAs), providing physical barriers to protect against pathogens, and modifying host immune function and inflammatory responses (Boleij & Tjalsma, 2012; Cong, Judge, et al., 2017; Quigley, 2013; Round & Mazmanian, 2009; W. Xu et al., 2017). Dysbiosis, the alteration or imbalance of the microbial community, disrupts human wellness and leads to disease in many different ways. It has been found to be associated with increased risk of colic (Kianifar et al., 2014) and necrotizing enterocolitis (NEC) in preterm infant (Cilieborg et al., 2016; Thomas, 2016), and can lead to infection, immune disorders (eg. inflammatory bowel disease) (Burcelin, 2016; Jiang et al., 2015; Marteau, 2009), metabolic syndrome (i.e., diabetes, obesity) (Menni et al., 2017; Rozanova, Voevodin, Stenina, & Kushnareva, 2002; Turnbaugh et al., 2009), neurological disorders (McKay et al., 2017; Tremlett et al., 2017), and cancer (Loo et al., 2017; Yamamoto & Matsumoto, 2016; Zhu, Gao, Wu, & Qin, 2013) later in life.

1.3.1 Gut Microbiota

The gut microbiota refers to the aggregation of microorganisms that reside in the GI tract. Compared to other parts of the body, the GI tract is the largest habitat for microbes, and it harbors 99% of the bacteria in the human body (Sender et al., 2016). The gut microbial community is a dynamic developmental ecosystem and the composition changes over time. The

colonization begins at birth and is influenced by the delivery mode, milk regimen, and gender (Cong, Judge, et al., 2017; Cong, Xu, Janton, et al., 2016; Cong, Xu, Romisher, et al., 2016; Thursby & Juge, 2017; P. Xu et al., 2017). Gut microbiome in infants is dominant by *Actinobacteria* and *Proteobacteria* and with relatively low diversity and a small number of operational taxonomic units (OTUs) (Backhed, 2011; Cong, Xu, Janton, et al., 2016; Rodriguez et al., 2015). During the first years of life and with the introduction of solid food, the gut microbiota gradually shifts, and matures to a community with increased diversity and adult-like composition. The gut microbiota in adults is relatively stable and is primarily dominated by *Firmicutes, Bacteroidetes, Actinobacteria*, and *Proteobacteria* (Khanna & Tosh, 2014). As the individual aging, the abundance of *Bacteroidetes* increases and becomes dominant in the gut microbial community (Rodriguez et al., 2015).

In addition to the shared changes caused by chronological age, the gut microbiota also displays variation among individuals and geographical population (Yatsunenko et al., 2012). Gut microbiota pattern is shaped by multiple factors, including colonization early in life, host genetics, diet, an individual's lifestyle, stress, incidence of diseases, and exposure to medications (Backhed et al., 2015; Cong, Judge, et al., 2017; Cong, Xu, Janton, et al., 2016; Cong, Xu, Romisher, et al., 2016; Dominguez-Bello et al., 2010; Hansen, Gulati, & Sartor, 2010; Karkman, Lehtimaki, & Ruokolainen, 2017; Kuang et al., 2017; Moles et al., 2013; Mosca, Leclerc, & Hugot, 2016; Tulstrup et al., 2015; W. Xu et al., 2017).

Gut microbiome plays a vital role in human's health. Gut bacteria produce enzymes, which ferment indigestible carbohydrates and generate short fatty acids, such as acetic, propionic, and butyric acids. These short fatty acids provide energy sources to host cell and are essential to cellular processes such as gene expression, proliferation, and programmed cell death

(Puertollano, Kolida, & Yaqoob, 2014). The microbiome also synthesize essential vitamins such as folic acid, Vitamin K and biotin and metabolize bile acids and xenobiotics, which are beneficial to the host (LeBlanc et al., 2013; Staley, Weingarden, Khoruts, & Sadowsky, 2017). In addition, gut microbiota has been found critical in the epithelial integrity and function. Dysbiosis of gut flora may lead to alteration in gastrointestinal motility, colonic epithelial cell homeostasis and impaired barrier function (Shi et al., 2017; Sommer & Backhed, 2016).

Moreover, gut bacteria influence the development of the systemic and intestinal mucosal immune systems (Thursby & Juge, 2017). Animal studies have found that germ-free animals have extensive deficits in the development of the gut-associated lymphoid tissues, antibody production and are more susceptible to certain bacterial infection (Round & Mazmanian, 2009). Alteration of the bacterial composition can affect the production and function of several cell types including epithelial cells, dendritic cells, and T cells, impact production capacity of inflammatory cytokines and lead to inflammation and disease (Hakansson & Molin, 2011; Schirmer et al., 2016).

1.3.2 Microbial translocation

Microbial translocation is the translocation of microorganisms and their products from the GI tract into the systemic circulation. Microbial translocation can occur in healthy individuals and increases when there is a dysfunction of the gastrointestinal barrier (Sandler & Douek, 2012). The alteration of the gut microbiome community causes intestinal mucosal barrier damage, GI immune dysregulation, which increases intestinal permeability and allows gut bacteria translocated to the bloodstream (Barlow, Yu, & Mathur, 2015; Mathur & Barlow, 2015) (Bischoff, 2017; Bouter, van Raalte, Groen, & Nieuwdorp, 2017; Chiriac, Mahapatro, Neurath, & Becker, 2017). Increased “leakiness” of the intestinal barrier further causes immune cell

activation and up-regulation of pro-inflammatory cytokines and drives immune perturbations and persistent systemic inflammation (Dinh et al., 2015). Microbial translocation has been recognized as an important pathogenic mechanism in many diseases. Microbial translocation-related persistent immune activation has been found associated with several infectious diseases such as HIV infection and hepatitis (French et al., 2013; Ortiz & Brenchley, 2018). Low-grade microbial translocation was also found associated with autoimmune and neurological disorders such as diabetes, multiple sclerosis and autism (Aravindhan, Mohan, Arunkumar, Sandhya, & Babu, 2015; Emanuele et al., 2010; Mirza & Mao-Draayer, 2017).

1.4 Immune Responses

Inflammation is the response of the body to harmful stimuli, such as injury and invasion of microorganism. When external stimuli invade the body, the immune system is activated, and the immune cells such as macrophages, mast cells, and circulating leukocytes rapidly recognize the signal derived from the pathogen invasion or cell damage by pattern recognition receptors (PRRs). There are different types of pattern recognition pathways, including pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). The receptors detect PAMPs and DAMPs, and initiate signaling cascades that trigger the release of inflammatory mediators, leads to vasodilation and increased blood flow. The increased permeability of blood vessels allows the migration of white blood cells from blood vessels to local sites and causes the cellular coagulation (Hakansson & Molin, 2011; J. Liu & Cao, 2016; Newton & Dixit, 2012).

PAMPs refer to the molecules that conserved within the microorganism but are not expressed in the host. Most common PAMPs are microbial nucleic acids, the bacterial protein flagellin, lipopolysaccharide (LPS), and endotoxin. In contrast, DAMPs are compounds that

released to the surface of the cell following the host cell damage. DAMPs include protein DAMPs and non-protein DAMPs. Examples of DAMPs include ATP, uric acid, heat-shock protein, the cytokine IL1α, and the calcium-modulated proteins S100A8 and S100A9 (calprotectin) (Newton & Dixit, 2012).

Inflammation is categorized as either acute or chronic. Acute inflammation occurs within hours following injury and is characterized by an accumulation of neutrophil and eosinophil granulocytes (Ward, 2010). Examples of acute inflammation are bacterial infection, the breakdown of cancer tumors and allergic reaction. Chronic inflammation refers to the process that involves lymphocytes, macrophages, and plasma cells and may last for months to years. In contrast to the resolution of acute inflammation, tissue healing and ongoing destruction occur simultaneously in chronic inflammation (Ward, 2010). Examples of chronic inflammation are persistent intra-cellular microbial infections (i.e., chronic HIV) and autoimmune disease (i.e., lupus).

Innate and adaptive immunity are two major categories of responses of the immune system. Innate immunity is general defense mechanisms that can be activated immediately when the stimuli entered into the body. Common elements involved in innate immune response include physical barriers such as skin and mucous membranes, substances in the blood, and immune cells such as neutrophil granulocytes, macrophages, and natural killer cells. Neutrophil granulocyte is one of the most critical factors in innate immune defense. Neutrophils regulate inflammation via the release of pro-inflammatory mediators, cytokines, and calprotectin (Rosales, Lowell, Schnoor, & Uribe-Querol, 2017). Calprotectin is DAMP with antimicrobial protective properties and can initiate and perpetuate the immune response (Smith & Gaya, 2012).

Adaptive immunity is an antigen-specific immune response. It recognizes and memorizes specific antigen and produces immune cells and antibodies that are specifically designed to attack that antigen. Adaptive immunity consists of B cells that produce antibodies and T cells that destroy pathogen-infected cells and produce cytokines that regulate the immune system. CD4 T lymphocytes play an important role in both humoral and the cellular part of the adaptive immune response. It is essential in protecting the body against pathogens. The depletion of the CD4 T lymphocytes is commonly related to the HIV infection and, especially with HIV progression and the absence of antiviral treatment (Baker et al., 2008). Dysfunction of the adaptive immune system may lead to major infections, and increase patients' risk of complications, morbidity, and mortality.

The immune responses are influenced by many factors including diet, age, stress, medication, and individual's genetics and medical condition. Recent research has suggested that both stress and gut microbiota have a significant impact on an individual's immune system (El Aidy, Dinan, & Cryan, 2014). Dysbiosis of gut microbial community leads to the invasion of pathogens to gut mucosa, release factors that disrupt pro- and anti-inflammatory balance, results in intestinal inflammation (El Aidy et al., 2014; Forbes, Van Domselaar, & Bernstein, 2016). Pathogens and their metabolites have been found associated with disrupted gut barrier function and increased gut permeability, which leads to the induction of toll-like receptors, ultimately activates immune responses (Carvalho, Aitken, Vijay-Kumar, & Gewirtz, 2012; Dinan & Cryan, 2012). Microbiota also influences the immune system by regulating the development of organized lymphoid structures and the function of immune cells such as T lymph cells (Emanuele et al., 2010). Furthermore, persistent pain/stress has been found related to dysregulation of innate and adaptive immune responses (Chapman et al., 2008; Dhabhar, 2014).

Stress can disrupt the immune function in many pathways, such as stimulating pro-inflammatory cytokine and suppressing its capacity to produce antibodies (Segerstrom & Miller, 2004). Dysregulated immunity caused by stress and dysbiosis is associated to a number of different diseases, such as infection, inflammatory bowel disease, multiple sclerosis, arthritis, lupus, asthma and cancer (Forbes et al., 2016; Reiche, Nunes, & Morimoto, 2004; Segerstrom & Miller, 2004).

1.5 Overall goal of the dissertation

Exist research findings have suggested that brain-gut-microbiota axis plays a critical role in regulating pain/stress, gut microbiome, and immune responses. Dysfunction of brain-gut microbiota has been found associated with several GI and brain disorders (Borre et al., 2014; Sekirov, Russell, Antunes, & Finlay, 2010). However, the underlying mechanisms of the interplay with each other have not been fully understood. This manuscript style dissertation investigated these mechanisms.

Pain/stress have significant influences on individuals' physical and psychological-behavioral health particularly in children (Schneiderman, Ironson, & Siegel, 2005). Exposure to prolonged stressors during infancy are associated with delayed neurobehavioral development, increased risk for cognitive deficits, mood disorders, aggressive behavioral problems, immune dysfunction, and physical and anatomic changes in the central nerves system (Cong, Wu, et al., 2017; Shaw, 2003). However, the majority of existing instruments were developed to measure acute pain using behavioral measures, which may not be able to capture the full effect of stress in preterm infants particular those are extremely immature. Study 1 was conducted to begin to develop an object instrument to quantitatively measure pain/stress in premature infants in the NICU.

Antiretroviral therapy (ART) has been a profound success in reducing the mortality and morbidity of HIV disease. However, chronic immune dysregulation remains a critical issue in HIV disease even in patients under viral suppressive ART treatment. To understand the underlying mechanism of long-term humoral immune perturbations, study 2 investigated the factors that contributed to the elevated anti-CD4 autoantibody production through brain-gut-microbiome axis model. The findings suggested that distinct systemic microbiome and levels of microbial translocation played a role in anti-CD4 autoantibody production in ART-treated HIV disease.

Preterm infants are vulnerable to severe infections (i.e., neonatal sepsis, necrotizing enterocolitis) and lead to up to 40 percent of mortality rate in the United States. Large bodies of evidence have indicated that stress (i.e., psychological, environmental and physical) can activate immune response, and cause immune dysregulation (Beversdorf, Stevens, & Jones, 2018; Y. Z. Liu, Wang, & Jiang, 2017). Early life stress stimulates the release of the proinflammatory cytokine, activate the HPA axis (O'Mahony et al., 2009). Elevated glucocorticoid hormone has been reported associated with decreases in the intestinal epithelial tight junction protein claudin-1, impairs epithelium homeostasis and barrier function (Zheng et al., 2017). The changes of intestinal barrier lead to the invasion of pathogenic microbes across the mucosal lining and activate the inflammation in the gut (Gareau, Silva, & Perdue, 2008; Power, O'Toole, Stanton, Ross, & Fitzgerald, 2014). The goal of study 3 was to test the hypothesized mechanism of cumulative pain/stress in modulating the gut inflammation among preterm infants.

1.6 Specific Aims and Hypothesis

Specific aim for study #1: To develop and validate the Accumulated Pain/Stressor Scale (APSS) to quantitatively assessing cumulative pain/stress in the neonatal intensive care unit

(NICU). The study 1 was the second stage of the scale development, which aimed to explore nationwide neonatal clinicians' perceptions regarding acuity and severity of each painful/stressful event in the NICU and to gather additional data to validate the APSS.

Specific aim of study #2: To investigate the underlying mechanisms of long-term humoral immune perturbations in HIV-infected patients. We hypothesized that microbial translocation of specific bacterial strains might play a role in B cell activation and anti-CD4 autoantibody production.

Specific aim of study #3: 1) To describe the gut inflammation in preterm infants during the first four weeks of life; 2) To explore the contributing factors that are associated with gut inflammation; and 3) To examine the association between cumulative stress in early life and gut inflammatory among preterm infants. We hypothesized that preterm infants with a higher score of cumulative pain/stress would have a higher level of Fecal calprotectin.

1.7 Conclusion

Dysregulation of immune function has a significant impact on human health and has been found related to infection, neurodegeneration, auto-immune disease and tumor genesis. The findings of each study presented in this dissertation will contribute to the understanding of the etiology and mechanism of immune activation through brain-gut-microbiota axis. Knowledge gained from the study will help to develop an individualized approach to restore immune-hemostasis and promote health in the vulnerable population.

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CHAPTER TWO

Original Article

Development of Accumulated Pain/ Stressor Scale (APSS) in NICUs: A National Survey

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Abstract:

High-risk neonates experience numerous painful/stressful procedures daily in neonatal intensive care units (NICUs). Accumulated pain and stress have a detrimental impact on infants' neurodevelopment. Few valid tools are available to measure accumulated pain/stressors among NICU infants. The aim of this study was to obtain nurses' perceptions about severity and acuity levels regarding each painful/stressful procedure that infants may experience in the NICU. The data will support developing a new instrument, the Accumulated Pain/Stressor Scale (APSS) in NICUs. A nationwide online survey was conducted through the U.S. National Association of Neonatal Nurses membership. Respondents were asked to rate the perceived severity of pain/stress associated with 68 procedures using a 5-point Likert scale and to categorize pain/stress as acute or chronic. Modal values were used to determine summary rankings among the procedures. Eighty-four neonatal nurses completed the survey. Among 68 procedures, nearly all were rated as painful/stressful to some degree. Five procedures (7%) had a modal value of five (extremely painful/stressful), nine (14%) had a value of four, 20 (29%) had a value of three, 30 (44%) a value of two, and four (6%) had a value of one (not painful/stressful). Forty-four procedures (65%) were perceived as acute, six (9%) as chronic, and 18 (26%) as both acute and chronic. Nurses' perceptions of pain severity and acuity regarding procedures in NICUs varied somewhat. Further studies are needed in developing and validating the scale. The development of the APSS can quantitatively measure the accumulated neonatal pain/stress.

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BACKGROUND

Evidence has established that newborn infants, including prematurely born infants, have the ability to perceive and experience pain. Moreover, because of immaturity of the descending pathways that inhibit pain impulses, preterm

neonates have lower tolerance for painful procedures than full-term infants, potentially leading to more severe consequences (Slater et al., 2010). In the high-technology, neonatal intensive care unit (NICU) environment, preterm infants are more likely to receive painful and stressful stimuli and, because of extended hospitalization, tend to receive it over a longer duration. On average, preterm infants experience 12-16 painful procedures per day (Carbajal et al., 2008). Growing concerns have been raised about accumulated and unmanaged pain/stress exposure in early life and its long-term, adverse consequences on the infant brain and neurodevelopment.

Vulnerable infants exposed to intense, repeated, or prolonged painful/stressful procedures in early life are likely to have altered pain pathways and thresholds, altered programming of stress systems, and impaired neurobehavioral outcomes, including cognitive, memory, and behavioral deficits, compared with full-term peers. These deficits may persist into adolescence and adulthood (Beauchamp et al., 2008; Grunau, Holsti, & Peters, 2006; Grunau et al., 2009; Hermann, Hohmeister, Demirakca, Zohsel, & Flor, 2006). When neonatal rats experience persistent peripheral inflammation, similar to multiple heelstick procedures given to human neonates, their spinal neuronal circuits exhibit changes in nociceptive primary afferent axons and show altered responses to sensory stimulation during adulthood (Bhutta et al., 2001; Ruda, Ling, Hohmann, Peng, & Tachibana, 2000). Similarly, repetitive or cumulative exposure to pain and stress is believed to permanently alter a human newborn's neuronal and synaptic organization (Anand & Scalzo, 2000; Fitzgerald & Beggs, 2001). More seriously, unrelieved excessive pain/stress can alter the structure and function of the developing brain in preterm infants through, for example, reduction of white and subcortical gray matter structures during maturation (Brummelte et al., 2012). These pain/stress exposures may be related to altered IQ in school-age children that is mediated by brain microstructural changes (Vinall et al., 2014). Strategies for assessing and managing cumulative pain/stress in the early life stage remain largely under-investigated and urgently need to be addressed.

More than 40 neonatal pain measurement tools currently are available for research and clinical use, but no single tool has been established as the "gold standard." Additionally, the majority of instruments were developed to measure acute pain across a short time period using physiological and behavioral cues, such as crying, facial expressions, heart rate variation, increased respiration, and decreased oxygenation (Cong, McGrath, Cusson, & Zhang, 2013), rather than directly quantifying cumulative pain and stress (Ranger, Johnston, & Anand, 2007).

Capturing bio-behavioral response to a painful procedure is challenging because the hypothalamic-pituitary-adrenal (HPA) axis in preterm neonates is not fully developed and physiological cues may not be displayed because of extreme immaturity (Newnham, Inder, & Milgrom, 2009). One study found that only 20% of extremely premature infants cry during a heel stick procedure (Gibbins et al., 2008a). Meanwhile, some physiological and behavioral indicators (e.g., heart rate and crying), may be nonspecific for pain and could be associated with other conditions, such as hunger or fatigue. Factors such as severity of illness, gestational age, frequency of and time since previous painful procedures, and medication use also may affect the ability of preterm infants to respond and could make behavioral variables ineffective in detecting neonatal pain (Gibbins et al., 2008b; Hatfield & Ely, 2015; Stevens et al., 2007).

These ambiguities leave neonatal nurses challenged by the issue of pain assessment in NICUs. Nurses working in NICUs have widely reported (e.g., 34.7% in the U.S. and 57.5% in China) that the pain assessment tools adopted in their units are inaccurate for measuring neonatal pain (Cong et al., 2014). Compared with acute pain, signs of repeated or cumulative pain tend to be more subtle in preterm infants, leading to under-recognition and undertreatment (Ranger et al., 2007). Young preterm infants may not display "appropriate" signs of pain response when they experience persistent painful or stressful procedures because they lack the energy reserves to express bio-behavioral responses (Ranger et al., 2007) or because the NICU experience has already led to abnormal brain development and to hypersensitivity to pain stimuli (Ranger & Grunau, 2014).

To determine the impact of repeated and cumulative pain and to provide an evidential basis for its treatment, NICU infants need to be monitored closely. The only tool that the authors found for assessing cumulative pain/stress in infants that is specifically targeted to the NICU setting is the Neonatal Infant Stressor Scale (NISS) developed in Australia by Newnham et al. (2009). The NISS provides a systematic way to identify and quantify infant stress. It lists 68 procedures and attaches a severity level to each one. However, in using the NISS, the authors found that it does not cover many painful events/stressors that occur as part of standard practice in NICUs in the United States.

To assess quantitatively and more accurately the extent and severity of pain/stress that infants experience in NICUs in the United States, the authors of

this paper developed the Accumulated Pain/Stressor Scale (APSS) in NICU. Because it is difficult to differentiate pain and stress in very preterm infants (Brummelte et al., 2015; Grunau et al., 2013), the authors use the term "painful/stressful" to characterize the full spectrum of events that can cause pain and produce stress in the neonatal population.

The initial version of the APSS consisted of 62 items (unpublished data) within nine categories of painful/stressful events potentially experienced by the NICU infants. The nine categories included blood draw, feeding, imaging, infection, peripheral venous access, central venous access, surgery, respiratory, and "miscellaneous." A focus group study was conducted to examine clinicians' perceptions regarding the concept of accumulated pain/stress and to obtain expert judgment of the content validity of the initial APSS scale. Nine neonatologists and five neonatal nurses were recruited from a level IV NICU (the highest level of NICU, with capacities to provide surgical repair of complex congenital or acquired conditions) in the northeastern United States and participated in the focus group discussion and completed a survey. Based on results from the focus group (unpublished), the APSS then was revised to a 68-item scale.

The current study is the second stage of the scale development, which reports findings from an evaluation of this revised, 68-item instrument. The objectives were to access a national sample of neonatal clinicians, to explore their perceptions about the acuity and severity of virtually all individual painful/stressful events that occur in NICUs in the United States, and to use that information to further document the validity of the APSS.

METHODS

Design

A descriptive, cross-sectional survey design was used in the study. American neonatal nurses were recruited through the National Association of Neonatal Nurses (NANN) webpage. Inclusion criteria for the participants were registered nurses over 18 years of age, English speaking, and working in a NICU setting in the United States at the time of survey.

Instrument

The survey questionnaire consisted of 68 painful/stressful procedures and events that commonly are experienced by neonates in the NICU. The procedures were grouped into 9 categories, including daily care, feeding, imaging, blood draw, peripheral venous access, central venous access, respiratory care, surgeries and major procedures, and infection. The participants were asked to classify each of the pain/stressors as acute, chronic, or both, and to use a numerical scale to indicate the severity level of each pain/stressor based on the participant's experience, knowledge, and judgment (1 = not painful/stressful; 2 = a little; 3 = moderate; 4 = very; and 5 = extremely painful/stressful). Survey respondents were instructed to draw conclusions from their experiences or memories of the NICU patient population rather than from the experiences of any particular infant.

Procedures

The study protocol was approved by the Institutional Review Board of the corresponding author's home institution. The survey was uploaded and administered electronically using Qualtrics Survey Software, Version 2015 (Provo, Utah). NANN members were e-mailed an invitation letter with a link to the Qualtrics survey through the NANN website. The information sheet with informed consent was posted on the survey website as the first question so that the potential participants were able to select "yes" or "no" to acknowledge that they had read the information sheet and gave informed consent before they could continue with the survey questionnaire. Demographic information was collected at the end of the survey questionnaire. After the initial invitation e-mail, two reminder e-mails were sent 10 days apart. The survey remained available for 30 days following the date of the initial e-mail to the participants. When participants completed the survey, their data were secured within a password-protected account. Once the survey was closed, the data were downloaded for statistical analysis.

Statistical Analysis

Survey data were analyzed using the R statistical software package (R 3.22). Descriptive statistical methods were used to summarize responses. This included calculating the mode and the average deviation from the mode (ADFM) for the acuity classification and for the severity ranking for each APSS item. The ADFM is the average of the absolute deviations from the mode. It quantifies dispersion or variability and can be used to describe agreement among the participants scoring each item. Correlation coefficients also were computed to evaluate associations among the severity of pain/stress that nurses rated for each procedure and their ages, educational levels, years of NICU experience, and highest NICU level in which they had worked.

RESULTS

Respondent Demographics

Eighty-four neonatal nurses from the United States participated in the national survey. The majority were female (98.8%), non-Hispanic white (95.2%), had a baccalaureate or higher degree (89.2%), and had worked in a Level III (capable of providing comprehensive care for infants born <32 weeks' gestation and weighing <1500 g and critically ill infants) or Level IV NICU (96.4%). The mean age of the neonatal nurses was 50.2 ± 10.8 years, with an average of 22.9 ± 10.9 years working experience in the NICU. Table 1 summarizes demographic characteristics of the participants.

Acuity of Perceived Pain/Stressors

Figure 1 depicts the acuity classification based on the mode of the acuity classifications for each item. Forty-four (64.7%) painful/stressful events were considered to be acute, including events from 8 of the 9 categories: blood draw, feeding, imaging, peripheral venous access, central venous access, procedures, respiratory, and miscellaneous. Six (8.8%) events were considered to be chronic: orogastric tube in situ (feeding), nasogastric tube in situ (feeding), peripheral venous access in situ (peripheral venous access), peripherally inserted central catheter (PICC) in situ (central venous access), nasal cannula (respiratory), and high frequency oscillatory (HFO)/jet ventilator with sedation (respiratory). Eighteen (26.5%) events from 6 categories (blood draw, feeding, infection, procedure, respiratory event, and peripheral venous access) were considered to be both acute and chronic.

The ADFM for the acuity of each event ranged from 0.02 to 0.93. The events with lowest ADFM (representing relatively high agreement among survey respondents) included cardiac echo (0.02), CAT scan and magnetic resonance imaging (MRI) (0.02), electrocardiogram (ECG) (0.08), ultrasound (0.10), and peripheral IV insertion with single attempt (0.11), whereas the ones with highest ADFM (representing lowest agreement) included peripheral IV insertion with multiple attempts (0.93), nasal continuous positive airway pressure (NCPAP) prong insertion (0.92), local infection (0.90), PICC line insertion with multiple attempts (0.87), and learning to bottle feed (0.83).

TABLE 1.
Demographic Characteristics of Responding Neonatal Nurses (N = 84)

Demographic Characteristics	Frequency	Percentage (%)
Age, years (n = 81)		
≤40	14	17.3
41-50	20	24.7
51-60	37	45.7
≥61	10	12.3
NICU practice, years (n = 83)		
≤10	16	19.3
11-20	17	20.5
21-30	26	31.3
≥31	24	28.9
Sex (n = 84)		
Male	1	1.2
Female	83	98.8
Race (n = 82)		
White	80	97.6
Asian	2	2.4
Ethnicity (n = 82)		
Hispanic	2	2.4
Non-Hispanic	80	97.6
Education (n = 84)		
Diploma/Associate	9	10.7
Baccalaureate	28	33.3
Master	35	41.7
Doctoral	12	14.3
Level of NICU (n = 84)		
Level II	3	3.6
Level III	43	51.2
Level IV	38	45.2

Severity of Perceived Pain/Stressors

Figure 1 also represents the mode and ADFN of the severity level of pain/stress for each event. Four (4.8%) events were ranked most frequently as 1 on the 5-point Likert scale (1 = not painful/stressful; 5 = extremely painful/stressful), 30 (35.7%) events as level 2, 20 (23.8%) events as level 3, 9 (10.7%) events as level 4, and 5 (6.0%) events as level 5. The four events that were scored as not painful/stressful were learning to breastfeed, oxygen tent, PICC line in situ, and removal from bed (wrapped). The events that were considered the most painful/stressful included gastroschisis abdominal content reduction, lumbar puncture, recovering from major surgery, chest tube insertion, and intubation with multiple attempts.

The ADFM for severity score ranged from 0.35 to 0.99 (Fig. 1). Examples of the events with lowest ADFM (and so with the strongest agreement among respondents) were oxygen tent (0.35), nasal cannula (0.35), cardiac echocardiography (0.37), chest tube insertion (0.38), and nasogastric/orogastric tube removal (0.38). Examples of the events with highest ADFM (and so the weakest agreement) were lumbar puncture (0.99), gastroschisis abdominal reduction (0.92), chest tube removal (0.85), tourniquet use for PICC line insertion (0.85), and conventional ventilation without sedation (0.77).

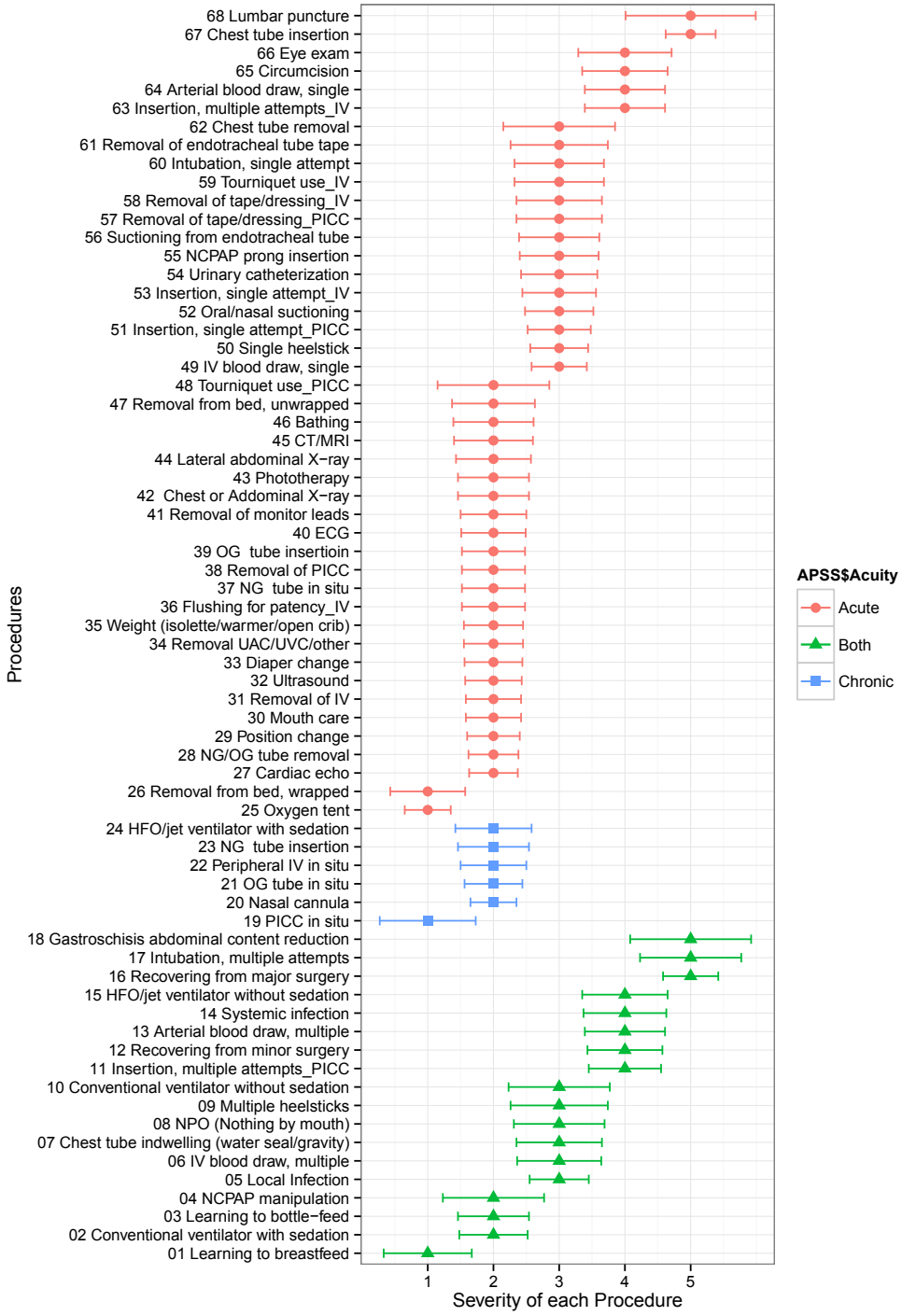

Figure 1. ■ Severity and acuity of each procedure. The graph shows mode (dot) and average deviation of mode (error bar) of the severity of each painful/stressful procedure on a 5-point scale. The procedures were grouped into acute (red), both acute and chronic (green), and chronic (blue) based on the mode of acuity.

For each participant, the mean severity score of all the events within each of the 9 categories was calculated. Spearman correlations between the mean score of each category and the demographic characteristics then were computed. The results showed no significant correlation between the mean perceived severity of any category and participants' age, years of experience as a neonatal nurse, or level of education.

However, there was a correlation between the highest NICU level where a participant had worked and the mean perceived pain/severity scores assigned to the blood draw category ($r = 0.249$, $p < .05$). Correlations between the mean score assigned to the blood draw category and the other 8 pain/stress categories were not statistically significant.

Summarizing Pain/Stressors Using the APSS

As explained above, the 68 painful/stressful events and procedures included in the APSS are classified into nine caregiving categories. Based on the survey results, within the nine categories, each item was assigned to a pain/stress severity level and to one of three acuity levels, acute (A), both acute and chronic (B), and chronic (C) (Table 2). To document infant pain/stressors using the APSS, clinicians can tally the acute (A) procedures, and tally and record the hours/minutes of both acute and chronic (B), or chronic (C) events, depending on the course of the events for each shift. The recorded frequency or duration of each event can be weighted through multiplication by the assigned pain severity level for that event. A total score for each acuity level (A, B, and C) can be calculated for each day by summing the weighted frequency or duration for each acuity level.

DISCUSSION

Neonatal nurses participating in this national survey perceived that almost all (64 of 68) of the procedures in the APSS were painful and stressful to neonatal infants to some extent. On average, the events and procedures included in the daily care, feeding, and imaging categories were perceived as only "a little painful/stressful." In contrast, procedures related to blood draw and medical procedures were considered "very painful/stressful." These results are consistent with findings from Newnham's study that procedures in nutrition, radiology, and nursing are perceived as less stressful, while medical procedures and surgery are perceived as most stressful (Newnham et al., 2009).

No correlations were found between nurses' demographic characteristics and the perceived pain/stress level for each category, except the relationship between the highest NICU level in which a nurse had worked and the nurse's perception of pain/stress level for blood draw. Nurses who have taken care of high-risk infants in a higher-level NICU viewed blood draw procedures as having a higher level of pain, a phenomenon that may be related to their experience or pain management training in such units.

Events that were classified as chronic by neonatal nurses tended to be less painful/stressful, with the modal severity level generally falling at 1 (not painful/stressful) or 2 (a little painful/stressful). Seventeen of 68 events were perceived as both acute and chronic by clinicians. One of the reasons for this result may be that there is still no established definition of chronic pain in the neonatal population (van Dijk & Tibboel, 2012). The concept of "pediatric chronic pain" has been defined as persistent or recurrent discomfort that lasts longer than an expected course of acute illness or injury, usually longer than 3 to 6 months (American Pain Society, 2012). However, this criterion usually cannot be applied to a neonatal population because the neonatal period is usually shorter than 3 months for most infants.

Although several studies have been conducted to conceptualize neonatal chronic pain, a consensus remains elusive (Pillai Riddell et al., 2009; van Ganzewinkel, Anand, Kramer, & Andriessen, 2014). Pillai Riddell's study listed potential examples of chronic pain in infancy, including repetitive acutely painful procedures such as cardiac patients with multiple surgeries, infants exposed to daily heel lances, and needle sticks (Pillai Riddell et al., 2009). van Ganzewinkel also concluded that experiencing "daily episodes of continuous or recurrent pain sensations" constitutes a potential etiology for chronic pain (van Ganzewinkel et al., 2014). The authors' findings are consistent with those of previous studies in that some events, including multiple heelsticks and other blood draws, were perceived as both acute and chronic because of initial high severity and long duration.

Agreement varied on the severity level perceived by neonatal nurses for individual painful/stressful events. Procedures that were viewed as less painful/stressful, such as oxygen tent, nasal cannula, cardiac echocardiography, and nasogastric/orogastric tube removal, tended to have higher agreement (small ADFM) among nurses, whereas procedures that were viewed as more painful/stressful, such as lumbar puncture, gastroschisis abdominal reduction, and conventional ventilation without sedation tended to have higher disagreement. This could be explained by the fact that no specific clinical circumstances for events in the APSS were provided to the survey respondents. In an open-ended question included at the end of the survey, 24 of the 84 participants commented on the lack of specific circumstances of the procedures when rating perceived pain/stress experienced by neonates. Multidimensional factors that may influence potential pain/stress responses and consequences include neonates' state and health conditions, the professional experience of the clinician who performs the procedure, whether comfort measures or analgesia is

given, and the diverse pain management techniques and protocols used in different units and hospitals. In light of the results, the more severe painful/stressful a procedure is, the more varied the clinical and caregiving factors that affect pain/stress assessment and outcomes. To prevent harmful long-term effects on infant health, clinicians need to address management of different levels of painful/stressful procedures in the NICU.

One of the limitations of this study is a failure to account fully for variability in pain/stress experience across different neonates' conditions and across different institutional contexts. However, the majority of existing pain measurement tools applied to the neonatal population, including assessments based on physiological and behavioral cues, has the same limitation (Krechel & Bildner, 1995; Newnham et al., 2009; Schiavenato et al., 2013; Stevens, Johnston, Petryshen, & Taddio, 1996). The current study attempted to moderate this limitation by using a national sample of NICU nurses in the expectation that including a diverse group of respondents would enhance validity and generalizability of the APSS. Differences in perceived pain/stress levels for each item that may be due to contextual factors were quantified and the mode was used to represent the severity level of each event under the majority of circumstances.

Another way in which the present study failed to account for differences in how neonates experience pain/stress in the NICU setting is that the survey questionnaire did not attempt to assess variation in nurses' perceptions of pain/stress among different postconceptional age groups. Mixed findings have been reported regarding the relationship of preterm infants' gestational and postnatal ages and their biobehavioral pain responses (Sellam, Cignacco, Craig, & Engberg, 2011). In one recent study, an infant's postmenstrual age was not found to be associated with any kind of behavioral or physiological pain responses (Sellam, Engberg, Denhaerynck, Craig, & Cignacco, 2013). Analogously, Newnham's study showed that the stress levels that were perceived by clinicians among different postconceptional age groups (<28, 28-32, and 32-37 weeks, respectively) were similar (Newnham et al., 2009).

Pharmacological and nonpharmacological interventions also are factors that affect the severity of each painful procedure and their roles as potential moderating factors were not reflected in the APSS. Pain interventions in the NICU still are not consistently used in clinical practice because of multiple factors, such as side effects of opioid medication, lack of standard pain protocols, and variability in clinicians' individual practices (Gibbins et al., 2015; Green, Darbyshire, Adams, & Jackson, 2014; Stevens et al., 2011). Therefore, further study is needed to calibrate the severity of painful events experienced by infants with concurrent administration of analgesic interventions.

Assessing cumulative pain and stress is critical and necessary in neonatal care given the strong association between repeated painful/stressful procedures in early life and subsequent adverse neurodevelopmental outcomes in preterm infants (Grunau, 2002; Provenzi et al., 2015). However, existing pain assessment tools mostly focus on acute, one-time pain events. To the authors' knowledge, only two instruments currently in use attempt to evaluate cumulative pain and stressors, the Neonatal Infant Stressor Scale (Newnham et al., 2009) and the Procedural Load Index (Schiavenato et al., 2013). Both tools were developed using experts recruited from local areas, which may limit their generalizability to NICUs in United States.

Implications for Nursing Practice and Research

This study describes the perception of pain intensity for the most commonly encountered procedures/events in NICUs and uses this information as part of the validation process for the APSS, an instrument designed to quantify cumulative pain/stress experienced by NICU infants. Based on the results, NICU nurses should be aware of the level of pain associated with each procedure/event as well as the cumulative pain/stress that infants experience daily. When sufficiently validated, the APSS scale will provide nurses guidance in delivering pain management for each infant and in advocating for infants to avoid unnecessary painful clinical procedures. Awareness of cumulative pain/stress scores also will provide NICU nurses the ability to identify infants with increased risk of adverse neurobehavioral development and to offer comforting measures, such as skin-to-skin contact, to promote more favorable developmental outcomes.

Existing evidence shows that cumulative pain/stress is strongly predictive of infants' neurobehavioral development. However, the lack of an established and accurate assessment tool increases the difficulty of future research exploring the undesirable consequences resulting from cumulative pain/stress or demonstrating the benefits of protective strategies that control or compensate for it. This study provides researchers a new approach for measuring cumulative pain/stress. With further validation, the APSS scale should be able to guide clinical practice for individual NICU infants and to support research that more conclusively documents effects of cumulative

pain/stress in preterm infants and the effectiveness of protocols for its management.

CONCLUSION

The new APSS scale will provide a systematic tool for evaluating cumulative pain/stress experienced by preterm infants in NICUs. By documenting pain/stressors using the APSS, clinicians can calculate the total frequency of acute (A) events and weighted scores for the severity of those events and the duration of both acute and chronic (B) or chronic (C) events, as well as durations that are weighted by severity. This information can guide the delivery or pain management and patient care, promoting neurodevelopmental outcomes later in life. Future studies are needed to further validate the APSS, especially expert judgment that addresses the impact of procedures/events with highly variable classifications by NICU nurses concerning chronicity or severity. In addition, larger studies need to be conducted applying the APSS scale in infant populations to test the reliability and validity of the instrument and, when necessary, to modify it in order to enhance its accuracy. Cut-off points for the scale that predict developmental consequences will also need to be explored and tested. With further support from the evidence, the APSS will be used to guide clinical pain practice and to support research into the effects of pain and its management.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.pmn.2016.08.004

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CHAPTER THREE

Distinct systemic microbiome and microbial translocation are associated with plasma level of anti-CD4 autoantibody in HIV infection

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Abstract

Microbial signals have been linked to autoantibody induction. Recently, we found that purified anti-CD4 autoantibodies from the plasma of chronic HIV-1-infected patients under viral-suppressed antiretroviral therapy (ART) play a pathologic role in poor CD4+ T cell recovery. The purpose of the study was to investigate the association of systemic microbiome and anti-CD4 autoantibody production in HIV. Plasma microbiome from 12 healthy controls and 22 HIV-infected subjects under viral-suppressed ART were analyzed by MiSeq sequencing. Plasma level of autoantibodies and microbial translocation (LPS, total bacterial 16S rDNA, soluble CD14, and LPS binding protein) were analyzed by ELISA, limulus amebocyte assay, and qPCR. We found that plasma level of anti-CD4 IgGs but not anti-CD8 IgGs was increased in HIV+ subjects compared to healthy controls. HIV+ subjects with plasma anti-CD4 IgG > 50 ng/mL (high) had reduced microbial diversity compared to HIV+ subjects with anti-CD4 IgG ≤ 50 ng/mL (low). Moreover, plasma anti-CD4 IgG level was associated with elevated microbial translocation and reduced microbial diversity in HIV+ subjects. The *Alphaproteobacteria* class was significantly enriched in HIV+ subjects with low anti-CD4 IgG compared to patients with high anti-CD4 IgG even after controlling for false discovery rate (FDR). The microbial components were different from the phylum to genus level in HIV+ subjects with high anti-CD4 IgGs compared to the other two groups, but these differences were not significant after controlling for FDR. These results suggest that systemic microbial translocation and microbiome may associate with anti-CD4 autoantibody production in ART-treated HIV disease.

Introduction

Chronic inflammation or immune dysfunction has been a critical issue in human immunodeficiency virus (HIV) disease even in patients under viral suppressive antiretroviral therapy (ART). ART significantly suppresses HIV viral replication, improves immune function, and decreases morbidity and mortality in HIV disease [1,2]. However, a substantial number of patients fail to reconstitute their peripheral CD4+ T cell counts even after long-term viral-suppressive ART treatment, and exhibit increased risks of complications, morbidity and mortality [3-7]. Previous studies have shown that thymic and lymphatic fibrosis, low nadir CD4+ T cell counts, sustained increases in inflammation, and microbial translocation may account for patients with poor CD4+ T cell recovery under viral suppressive ART treatment [5,8-21]. However, the exact mechanism governing poor CD4+ T cell recovery is still unknown. In our recent work, we studied the anti-CD4 autoreactive IgGs purified from plasma of ART-treated aviremic patients with peripheral CD4+ T cell counts less than 350 cells/μL. Our study has shown that anti-CD4 autoreactive IgGs induce CD4+ T cell death through antibody-mediated natural killer (NK) cell cytotoxicity *in vitro*, suggesting that anti-CD4 autoantibodies play a role in blunted CD4+ T cell reconstitution after ART treatment [22]. Consistently, we have found that purified NK cells from patients with blunted CD4+ T cell recovery were enriched in cytotoxic cells and were able to mediate uninfected CD4+ T cell death *ex vivo* [23].

Prior to ART treatment, HIV infection results in significant B cell depletion, especially memory B cell depletion, B cell hyperactivation and heightened plasma levels of autoantibodies, as well as impaired vaccine responsiveness [24-28]. These B cell perturbations cannot be completely

explained by the lack of contribution from CD4+ T cells; B cell intrinsic defects have been observed [29,30]. For example, our previous work has shown that purified B cells from HIV-infected subjects had reduced proliferation capacities in response to toll-like receptor (TLR) 9 ligand stimulation compared to B cells from healthy controls *in vitro* [30]. Another study from Moir's group reported that purified B cells from HIV-infected patients had reduced antigen-presenting function compared to B cells from healthy controls when co-culturing with purified T cells from the same healthy donors [29]. These results suggest B cell intrinsic dysfunction in HIV disease. Furthermore, B cells have been reported activated even after long-term viral-suppressive ART treatment, which may account for inconsistent serologic antibody responses and cellular responses in patients given seasonal influenza vaccination [31].

The underlying mechanisms of long-term humoral immune perturbations in HIV-infected patients, despite undergoing ART treatment, are still largely unknown. The fecal microbiota and microbial translocation from the gastrointestinal (GI) tract to systemic circulation have been recently investigated as a major driver of immune perturbations and persistent systemic inflammation in HIV disease [32-35]. Increased intestinal permeability due to mucosal barrier dysfunction, GI immune dysregulation and/or altered intestinal microbiome are considered to be significant factors related to microbial translocation and HIV pathogenesis. Differences in fecal microbiome in HIV-infected patients versus healthy controls are associated with systemic inflammation [32]. Mechanistically, microbial products such as TLR ligands can induce autoantibody production and may play a pathogenic role in autoimmune diseases [36-38]. Increased systemic microbial translocation and its associated inflammation may result in B cell hyperactivation and perturbation in HIV disease. After long-term repeated stimulation by low

concentrations of TLR ligands (compared to one dose and high concentration as vaccine adjuvants) and other microbial products released from the gut [24-26,39], B cells may be polyclonally activated as reflected by increased total IgM and IgG [26,40].

In the current study, we hypothesize that microbial translocation of specific bacterial strains may play a role in B cell activation and anti-CD4 autoantibody production. We, therefore, investigate systemic bacterial microbiome, the magnitude of microbial translocation, and plasma anti-CD4 autoantibodies in HIV+ subjects under long-term viral suppressive ART treatment.

Methods

Study Design, Subjects, and Data Collection

This study was approved by the Institutional Review Board at Medical University of South Carolina. All methods were performed in accordance with the relevant guidelines and regulations. All participants provided written informed consents. In the present study, 12 healthy volunteers and 22 HIV+ ART-treated aviremic (plasma HIV RNA < 50 copies/mL) patients were enrolled. The clinical characteristics of participants are shown in Table 1.

Inclusion and exclusion criteria

All participants were age 18 years and older. All patients had documented HIV infection and were receiving a stable antiretroviral regimen with plasma HIV RNA < 50 copies/mL more than

two years prior to study entry. Transient viremic blips did not exclude participation if flanked by viral levels below detection limits. Exclusion criteria included pregnancy, breast-feeding, surgery, chemotherapy, inflammatory bowel diseases, and uses of steroids more than 10 mg per day for more than 120 days or uses of antibiotics within 14 days prior to enrollment.

ELISA for detection of anti-CD4 IgGs and anti-CD8 IgGs

Human soluble CD4 protein (sCD4, Progenics Tarrytown, NY) or human soluble CD8B/P37/LEU2 protein (sCD8, Sino Biological Inc. Beijing, China) were diluted at the concentration of 16 μg/ml and added to microtiter wells, and incubated at 4°C overnight. Microwells were washed three times with phosphate buffered saline wash buffer (PBS with 0.1% Tween 20), and then blocked with PBS containing 3% bovine serum albumin (BSA) for 120 min at 37°C. Plasma was diluted 1:40 in PBS containing 3% BSA and 100 μl of the dilution were added to the wells. The plate was incubated at room temperature for 60 min. Biotin-labeled goat anti-human IgG was added at 1:5000 dilution in PBS containing 3% BSA. The plate was then incubated for 60 min at room temperature. Horseradish peroxidase conjugated streptavidin (HRP-Streptavidin) was added at a 1:1000 dilution in PBS containing 3% BSA, and then incubated for 30 min at room temperature. After washing, 100 μl 2, 2'-Azino-di (3-ethylbenzthiazoline-6-sulfonate) were added and incubated for 30 min, and 405 nm emission was read within 30 min. PBS containing 3% BSA alone was used as a negative control and anti-CD4 and anti-CD8 antibodies were used as positive controls.

The 40th percentile (50 ng/mL) of anti-CD4 IgG was used to define the cutoff for high and low levels of the IgG. Therefore, patients with plasma anti-CD4 IgG level above 50 mg/mL was defined as the high anti-CD4 IgG group; and patients with plasma anti-CD4 IgG level equal or below 50 ng/mL was defined as the low anti-CD4 IgG group.

Plasma levels of LPS, soluble CD14 (sCD14), LPS binding protein (LBP)

Plasma samples were collected into tubes containing EDTA and stored at −80°C until they were thawed once. The method was descripted from our previous studies [41-43]. Briefly, the plasma samples were diluted to 10% with endotoxin-free water, and LPS was quantified using a commercially available limulus amebocyte assay kit (Lonza Inc., Allendale, NJ) according to the manufacturer's protocol. sCD14 and LBP were quantified using kits from R&D (Minneapolis, MN) and Hycult Biotech (Plymouth Meeting, PA) respectively following manufacturers' protocols.

Quantitative polymerase chain reaction (PCR) for measurement of bacterial 16S rDNA

DNA was extracted from 400μL endotoxin free water and 400 μL plasma using QIAamp UCP pathogen Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The method was descripted from our previous studies [31,41]. Briefly, a 20 μL amplification reaction consisted of 10 μL of 2x Perfecta qPCR ToughMix (Quanta, Gaithersburg, MD), 0.3 μmol/L forward and reverse primers, 0.175 μmol/L probe (338P: 5'-FAM-GCTGCCTCCCGTAGGAGT-BHQ1− 3'), and 5 μL of the template plasma DNA. Degenerate forward (8F: 5'-AGTTTGATCCTGGCTCAG-3') and reverse (515R: 5'-

GWATTACCGCGGCKGCTG-3') primers were used to amplify DNA templates encoding 16S rRNA. The DNA was amplified in duplicate, and mean values were calculated by subtracting values in the water control. A standard curve was created from serial dilutions of plasmid DNA containing known copy numbers of the template. The reaction conditions for amplification of DNA were 95°C for 5 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min [41].

Plasma microbial DNA extraction, sequencing and data process

Microbial DNA extraction was described above in 16S rDNA assay. The 16S rRNA gene V4 variable region PCR primers 515/806 with barcode on the forward primer were used in a 30 cycle PCR (5 cycle used on PCR products) using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together (e.g., 100 samples) in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. Then the pooled and purified PCR product was used to prepare the DNA library by following Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines.

The Q25 sequence data derived from the sequencing process was processed using a proprietary analysis pipeline (www.mrdnalab.com, MR DNA, Shallowater, TX). Sequences were depleted of barcodes and primers, then short sequences < 200bp and sequences with ambiguous base calls, and sequences with homopolymer runs exceeding 6bp were removed. Next, sequences were denoised and operational taxonomic units (OTUs) were defined clustering at 3% divergence (97% similarity) followed by removal of singleton sequences and chimeras [44-48]. Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes, RDPII and NCBI [49]. The data has been summarized at each taxonomic level by both raw counts and relative abundances. For each plasma sample and water control, absolute and relative abundance in OTU tables were generated. To control for contamination, two water samples were used as negative controls for DNA extraction. ß-diversity is different from the samples of patients, healthy and water controls (Supplemental figure 1). In the data analysis, we used both method of subtracting the mean abundance of the OTUs and removing any OTUs that are present in the water control. The PERMANOVA variability from both methods are the same. The results in this paper were presented based on method of removing mean absolute abundance of OTUs. See supplemental table 1 for the raw data of the read counts and relative abundance from each sample including water controls.

Statistical Analysis

In the pre-specified hypothesis, we were interested in the comparisons of HIV+ high anti-CD4 antibody group versus HIV+ low anti-CD4 antibody group or healthy controls; therefore, P values from comparing HIV+ high anti-CD4 antibody group to each control group were not

adjusted for multiple comparisons [50]. Non-parametric Mann-Whitney U tests were applied to the current study.

For microbiome analysis, OTU tables and different levels of taxonomy tables derived from the sequencing process described above were imported to R (version 3.3.1) for statistical analysis [51]. The mean values of two negative controls were subtracted from each sample's OTU to control for the contamination. Simpson index of diversity was calculated using Vegan package [52] measure α diversity of each sample. Spearman's Correlation test were used to assess the association among Simpson diversity index, clinical and demographic characters and autoreactive antibody. Bray-Curtis and Jaccard dissimilarity were calculated using Vegan package to evaluate ß-diversity, the compositional dissimilarity among the microbial community. Jaccard dissimilarity measures the dissimilarity between samples based on the presence/absence of the data, whereas Bray-Curtis dissimilarity was calculated based on both presence/absence and abundance. The relationships between ß-diversity of the microbial community and autoreactive antibody titer were assessed using PERMANOVA in Vegan package. Analysis of indicator species (Indicspecis package) was used to assess the relationship between the occurrence/abundance of species at the genus level with different clinical characters. False discovery rate (FDR) correction was applied to control for multiple comparisons.

Results

A total of 34 participants completed the study, including 22 HIV patients and 12 healthy controls. Demographic characteristics of the participants are illustrated in Table 1.

Plasma anti-CD4 IgG level but not anti-CD8 IgG level was increased in aviremic ART-treated HIV+ subjects compared to healthy controls

Following our recent work, we investigated the mechanism of anti-CD4 autoantibody production in well-controlled ART-treated HIV infection. We first analyzed plasma levels of anti-CD4 IgG as well as anti-CD8 IgG in age-matched healthy controls and aviremic ART-treated HIV-infected subjects. We found that the plasma level of autoreactive anti-CD8 IgG was similar in controls and HIV+ subjects, but the level of anti-CD4 IgG increased in the HIV+ subjects compared to controls (Figure 1A-1B), suggesting that B cell function is still abnormal even after long-term ART treatment and successful viral suppression.

Plasma microbial translocation was elevated in HIV+ subjects with high plasma anti-CD4 IgGs compared to healthy controls

Next, to investigate the association of systemic microbial translocation and plasma anti-CD4 IgGs level in HIV-infected subjects, we stratified patients to either high plasma autoantibody level or low plasma autoantibody level group. The cutoff value of 50 ng/mL plasma anti-CD4 IgG was defined based on 40 up-percentile, and no healthy controls were above that value. Notably, both plasma LPS level and bacterial 16S rDNA level, markers of microbial translocation [41], tended to increase in HIV+ subjects with plasma anti-CD4 IgG below 50 ng/mL compared to healthy controls but have not achieved significant differences (Figure 1C-1D). Importantly, HIV+ subjects with high plasma level of anti-CD4 IgGs exhibited significantly

elevated plasma microbial translocation (Figure 2), suggesting that residual increased systemic microbial products may be associated with autoantibody production. In addition, we have evaluated the other two markers related to microbial translocation, sCD14 and LBP in plasma. Indeed, HIV+ subjects with high anti-CD4 IgGs had increased plasma sCD14 (Figure 1E) and LBP (Figure 1F) levels compared to the other two study groups. These results suggest that HIV+ subjects with high plasma anti-CD4 IgGs, but not HIV+ subjects with low plasma anti-CD4 IgGs, had increased systemic microbial translocation compared to healthy controls.

Distinct plasma microbial profiles in HIV+ subjects with high anti-CD4 IgGs compared to controls

To investigate the difference of microbial translocation in healthy controls and HIV+ subjects, we performed and analysed plasma microbiome (Figure 2A-2E). The samples yielded a total of 1,218,338 reads with an average of 34758.15 (±15380.71) reads per subject and 18280.5 (±10127.89) reads for water control. A total of 2408 OTUs were found in samples of all 34 subjects. On average, 400 (± 98) OTUs were found in each sample. In contrast, 439 OTUs (average 272 ± 76) were found in the water control, and the top phyla were *Proteobacteria (*79.3%), *Firmicutes (*12.5%), *Deinococcus-Thermus (*7.3%), *Cuampbacteroa* (0.7%) and *Actinobacteria* (0.2%). In the phylum levels among all samples, 57.4% were *Proteobacteria,* 19.2% were *Firmicutes*, 10.5% were *Actinobacteria,* and 6.4% *Bacteroidetes* in plasma (Figure 2A). A decreased ratio of *Firmicutes/Bacteroidetes* was reported on the fecal microbiome in autoantibody-derived autoimmune disease such as systemic lupus erythematosus (SLE) [53,54]. In this study, the ratios of *Firmicutes/Bacteroidetes* were 0.58±0.45 in healthy controls, 0.37±0.38 in the low anti-CD4 IgG HIV+ subjects, and 0.32±0.30 in the high anti-CD4 IgG HIV+ subjects,

respectively, but did not achieve significant difference between any two groups (mean±SD, P > 0.05). At the class level, *Gammaproteobateria, Betaproteobacteria, Bacilli* and *Alphaproteobacteria* were predominant (80.3%) in the low anti-CD4 IgG group (Figure 2B). Notably, the plasma enrichment of *Alphaproteobacteria* class was significantly higher in the low anti-CD4 IgG patient group compared to the high anti-CD4 IgG patient group after controlling for FDR ($t = 3.22$, $P < 0.05$, Figure 2B). At the family level, *Staphylococcaceae* and *Pseudomonadaceae* were increased in the high anti-CD4 IgG patient group compared to the other two groups (Figure 2D). At the genus level, *Alicycliphilus, Pseudomonas,* and *Staphylococcus* had increased relative abundance in the high anti-CD4 IgG patient group compared to the low anti-CD4 IgG patient group. (Figure 2E). Although the microbial components were different from the phylum to genus levels in HIV+ subjects with high anti-CD4 IgGs compared to the other two groups, these differences were not significant after controlling for FDR.

Reduced plasma microbial diversity was associated with increased plasma anti-CD4 antibodies in HIV-infected individuals

Next, to investigate the difference of composition in plasma microbiome in the three study groups, we analysed microbial diversity including Simpson Diversity Index, Shannon index and species number observed. The Simpson and Shannon diversity indexes in the high anti-CD4 IgG HIV+ subject group were significantly lower compared to the low anti-CD4 IgG HIV+ subject group ($P = 0.04$ and $P = 0.05$ respectively, Figure 3A and 3B). The numbers of species were 365.8±99.6 in healthy controls, 373.5±91.2 in the low anti-CD4 IgG HIV+ subjects, and

362.1±85.7 in the high anti-CD4 IgG HIV+ subjects, respectively (mean±SD, $P > 0.05$). There was an inverse correlation between plasma anti-CD4 IgG level and the Simpson diversity index in HIV+ subjects but not in healthy controls (Figure 3C and 3D). Moreover, ß-diversity, the compositional dissimilarity among the microbial community was assessed using nonmetric dimensional scaling with both Bray-Curtis Coefficient and Jaccard Index, and revealed significant clusters between HIV-infected subjects with plasma anti-CD4 IgG level > 50 ng/mL and their counterparts (Figure 4). Nonetheless, anti-CD4 IgG level explained 6.8% of the variation of Bray-Curtis coefficient among HIV-infected individuals after controlling for plasma LPS level, duration of the ART treatment and CD4 counts (PERMANOVA, $n = 22$, $P < 0.05$); PERANOVA test of anti-CD4 IgG level on Jaccard Index yielded a similar result. Indicator species analysis showed that patients who had a higher level of anti-CD4 IgG (> 50 ng/mL) had significantly higher levels of *Alicycliphilus* ($P < 0.05$) and *Hylemonella* ($P < 0.05$). However, the significances disappeared after controlling for FDR.

Discussion

Increased levels of autoreactive antibodies or autoimmune diseases have been shown in HIV/SIV infection [55-62]. ART treatment reduces B cell hyperactivation [63]. Our recent study shows that anti-CD4 autoantibodies purified from plasma of immunologic non-responders (undetectable plasma viral load, ART-treated, and CD4+ T cell counts < 350 cells/μL) mediated CD4+ T cell death through antibody-dependent NK cell cytotoxicity, suggesting that anti-CD4 IgG plays a role in poor CD4+ T cell recovery under viral suppressive ART treatment [22]. In the current study, we

found that both quantity and quality of plasma microbial products in ART-treated HIV-infected subjects was associated with anti-CD4 autoantibodies.

Microbial TLR and its agonists play a role in autoantibody production and autoimmune diseases [64,65]. Our previous study showed that plasma level of TLR4 ligand LPS was associated with inflammation and B cell activation in HIV disease [43]. Although ART treatment greatly reduces cell apoptosis and activation and thus limits autoantibody production [43,66-69], we found that anti-CD4 specific antibody is a key exception (Figure. 1A). Moreover, altered B cell receptor (BCR) and TLR signals (e.g., MyD88) may promote autoreactive B cell selection [70]. Indeed, HIV+ subjects had elevated levels of microbial translocation (Figure. 1C-1D) and cycling B cells [31] compared to healthy controls, implying that bacterial products (e.g., LPS) may play a role in activating B cells. Nonetheless, how microenvironmental and inflammatory factors drive the breakdown of B cell tolerance, especially in humans, is not fully understood. Notably, autoimmune diseases in HIV are often observed after ART [55,71,72], implying that pathologic autoantibodies are developed post the ART treatment.

Interestingly, a diverse bacterial DNAs were found in the plasma of healthy controls (Figure 2). These findings are consistent with the study from Païssé S [73]. Low levels of microbial translocation occur in healthy individuals but increase when there is a GI barrier disruption. On the other hand, dysbiosis of gut microbiome community may result in mucosal immune dysfunction and intestinal mucosal barrier damage, which allows gut microbial translocation to the bloodstream [74,75 76-78]. Increased "leakiness" of microbial products (e.g., LPS) from the intestinal barrier further may cause systemic immune cell activation and drives immune

perturbations [32]. Intestingly, we observed a trend decrease in the *Firmicutes/Bacteroidetes* ratio in HIV+ subjects with high anti-CD4 IgG level compared to the other two groups, which is consistent with prior reports on the fecal microbiome in autoimmune disease such as systemic lupus erythematosus (SLE) [53,54].

Most microbiome studies used stool, saliva, or cervical-vaginal lavage fluid samples, very rare study was done on plasma microbiome due to highly technical demands [32,79-81]. A recent study reported that HIV-infected patients had different fecal microbial community composition compared to healthy controls [32]. Fecal microbiome from HIV-infected patients was enriched in *Enterobacteriales, Erysipelotrichaceae, Proteobacteria, Enterobacteriaceae, Gammaproteobacteria, Erysipelotrichi, Barnesiella*, and *Erysipelotrichales*, but was depleted in *Rikenellaceae* and *Alistipes*, relative to healthy controls [32]. Another study showed that HIV-infected patients with low peripheral CD4+ T cell counts exhibited reduced enteric bacterial diversity, which is consistent with our findings [79]. Both studies indicate that enrichment of *Enterobacteriaceae* was associated with systemic inflammation [32,79]. Consistently, plasma enrichment of *Proteobacteria, Gammaproteobacteria* and *Betaproteobacteria* was also observed in HIV+ individuals compared to healthy controls in the current study, but the difference did not achieve statistical significance (Figure 2). However, we did not observe enrichment in other bacteria products reported in the fecal microbiome study besides *Proteobacteria, Gammaproteobacteria* and *Betaproteobacteria* in plasma from HIV+ individuals relative to healthy controls [32]. Nonetheless, it is important to investigate microbiome simultaneously in plasma and mucosal sites in HIV in the future.

TLR4 signaling was increased with transgenic mice for a TLR chaperone molecule (gp96), which resulted in a lupus-like autoimmune glomerulonephritis (26). Flares of autoimmune diseases have been observed with infection [82] in humans and also is a inducer of autoimmunity in mice. Decreased anti-dsDNA antibodies were observed in TLR2 and TLR4 knockout C57BL/6 (lpr/lpr) mice; and autoantibodies were induced by LPS stimulation through TLR4-dependent cell signaling pathway in lupus-prone mice [83,84]. Therefore, increased bacterial product translocation may play a key role to induce autoantibodies in HIV. However, the association of plasma bacterial products (e.g., LPS) and anti-CD4 IgG level we observed in the current study does not prove causality. Next, we will give HIV-infected humanized animal models with specific bacterial products (e.g., LPS) found in plasma of the high HIV+ subjects to evaluate anti-CD4 autoantibody production. The other possibility of this association can be high anti-CD4 autoantibody-mediated immunodeficiency (poor CD4+ T cell recovery [22]) and increased inflammation favor particular bacterial survival. Furthermore, plasma soluble CD4 level was similar among the three study groups [22], suggesting that increased anti-CD4 IgG in some patients may not result from increased antigens in plasma. However, we do not know whether the level of CD4 antigen and HIV proteins (e.g., gp120 [85]) with CD4 binding capacity is increased in lymph nodes, raising the question that increased anti-CD4 IgG may be due to increased antigens in the patients with high anti-CD4 IgG level.

Women in general have higher humoral and cellular immune responses relative to men, as well as higher prevalence of autoimmune diseases [86]. Mechanisms accounting for sex differences in autoimmune diseases include sex-induced breaks in tolerance and increases in peripheral cell activity, such as TLR responsiveness, T regulatory cells, environmental and genetic factors [87-90].

Consistently, we found that there were more women in the high HIV+ anti-CD4 autoantibody group compared to the low HIV+ anti-CD4 autoantibody group (Table 1). Whether anti-CD4 autoantibody induced by female sex hormones or sex hormone-mediated immune responses is worth further investigation.

This is the first study to date to report plasma microbiome and microbial products (e.g., LPS) in relation to autoantibodies in HIV patients. One of its limitations remains a small sample size. Due to the small sample size and large amount of microbial species observed in the plasma, most significant differences of microbiome among the study groups were not demonstrable after FDR correction. Another limitation is that other factors that may influence gut microbiota composition and bacterial translocation, such as diet, usage of probiotics and antibiotics, and the comorbidity of the patients were not controlled in the study. Therefore, the interpretation and generalization of findings may be limited. Future studies with large and diverse sample sizes are needed to lead a greater understanding of the concept of microbial translocation and auto-immune responses. In addition, the contributing factors for microbiome including sex should be considered.

In summary, we found that elevated plasma anti-CD4 IgG in HIV-infected subjects was associated with the magnitude of systemic microbial translocation and systemic microbiome. At the class level, *Gammaproteobateria, Betaproteobacteria, Bacilli* and *Alphaproteobacteria* were predominant in the low anti-CD4 IgG group. At the genus level, *Alicycliphilus,* and *Hylemonella* had elevated relative abundance in the high anti-CD4 IgG patient group compared to the low anti-CD4 IgG patient group. These results suggest that systemic microbial translocation and

microbiome may play a role in anti-CD4 autoantibody production in HIV infection. However, the small sample size in the current study prevents us to draw further conclusions.

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Additional Information

Accession codes: The data are available at the NCBI Sequence Read Archive (SRA) under accession no. SRP120355 (http://www.ncbi.nlm.nih.gov/sra).

Author Contribution

WX: Wrote the first version of manuscript and analyzed the microbiome data
ZL: Performed experiments
AA: Analyzed the microbiome data and assisted statistical analysis
LM: Recruited donors and helped study design
ZW: Statistic data analysis
BL: Designed the study and revised manuscript
ZQ: Designed the study and revised manuscript
SH: Designed the study and revised manuscript
KM: Analyzed the microbiome data and designed the study
XC: Analyzed the microbiome data, revised manuscript and designed the study
WJ: Designed the study and revised manuscript

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Figure legends

Figure 1. Plasma level of anti-CD4 IgG and its association with microbial translocation in HIV+ subjects. sCD4 and sCD8 proteins were used to detect plasma anti-CD4 IgGs (A) and anti-CD8 IgGs (B) by ELISA. Plasma levels of LPS were detected by limulus amebocyte assay (C), bacterial 16S rDNA were detected by qPCR (D), sCD14 (E) and LBP (F) by ELISA in healthy controls and HIV+ subjects with plasma anti-CD4 IgG > 50 ng/mL and ≤ 50 ng/mL. Non-parametric Mann-Whitney tests.

Figure 2. Circulating microbiome relative abundance analysis in healthy controls and HIV+ subjects. Microbial DNA was extracted from plasma and V4 variable region of bacterial 16S rDNA gene was amplified. The relative abundance of phylum (A), class (B), order (C), family (D), and genus (E) level bacteria (> 1%) were shown in plasma from healthy controls, HIV+ subjects with plasma anti-CD4 IgG level ≤ 50 ng/mL and HIV+ subjects with anti-CD4 IgG > 50 ng/mL. The plasma enrichment of *Alphaproteobacteria* class was significantly higher in the low anti-CD4 IgG patient group compared to the high anti-CD4 IgG patient group after controlling for FDR.

Figure 3. Reduced diversity was associated with increased plasma level of anti-CD4 autoantibody in HIV+ subjects. Box and whiskers plots of the Simpson (A) and Shannon (B) diversity indexes of plasma samples from HIV+ subjects with anti-CD4 IgG levels ≤ 50 ng/mL, > 50 ng/mL and health controls. The top and bottom boundaries of each box indicate the 3rd and 1st quartile values, respectively. The central horizontal line represents the median values. The dot

represents Simpson and Shannon diversity index of each sample. Non-parametric Mann-Whitney U tests. Correlations between the Simpson diversity index and plasma anti-CD4 IgG levels in healthy controls (C) and HIV+ subjects (D). Spearman correlation tests.

Figure 4. Nonmetric multidimensional scaling ordination (NMDS) plot of the OTUs with fitted vectors of clinical variables (A), and based on the abundance of bacterial phyla (B). Dots with different colors represent data from each plasma sample in HIV+ subjects with anti-IgG level ≤ 50 ng/mL (red) and HIV+ subjects with anti-CD4 IgG > 50 ng/mL (green). Ellipses denote the standard deviation of weighted average NDMS score of anti-IgG level ≤ 50 ng/mL group (red) and anti-CD4 IgG > 50 ng/mL group (green). Community differences were verified by PERMONOVA test (Adonis, $P < 0.05$). Arrows represent the direction and magnitude of correlation of each clinical variable (A) and the abundance of bacterial phyla (B) with the ordination axes.

Table 1. Demographic and clinical characteristics of the participants

	Healthy control	HIV+/αCD4^{low}	HIV+/αCD4^{high}	P1	P2	P3
Number	12	13	9			
Age	43.5 (33.5-56)	43 (26-46.5)	47 (36-56.5)	0.25	0.77	0.21
Gender (Male/%)	3 (25%)	11 (84.6%)	3 (33.3%)	0.005	>0.99	0.04
Race (AA/%)	7 (44%)	8 (57%)	7 (58%)	0.72	0.7	0.52
Nadir CD4 count (cells/μL)		361 (226-490)	229 (124-426)			0.19
Duration of ART (yr)		4 (3.5-6.5)	6 (4-6)			0.82
CD4 count (cells/μL)	828 (523-1043)	634 (514-744)	450 (321-677)	0.50	0.07	0.07
%ki67+ CD4	1.0 (0.7-1.6)	2.8 (1.9-3.8)	2.5 (1.7-3.9)	< 0.0001	0.001	0.73
%annexin V+ CD4	19 (13.5-37.7)	29.4 (27.1-43)	26.9 (15.7-32)	0.14	0.60	0.18
B cell count (cells/μL)	219 (112-235)	239 (132-314)	185 (130-245)	0.29	0.65	0.37
%ki67+ B cells	0.9 (0.7-1.1)	1.5 (0.9-2.0)	1.3 (0.9-2.7)	0.03	0.03	0.84
%annexin V+ B cells	9 (5.5-18)	19.4 (12.6-27.8)	17.6 (13.3-33)	0.007	0.04	0.86
Plasma soluble CD4 (ng/mL)	2.1 (1.2-4.9)	1.7 (0-2.7)	1.8 (0.3-2.7)	0.37	0.39	0.66
Current ART regimen						
Multi-Class Combination		11 (84.6%)	4 (44.4%)			>0.99
NRTIs		2 (15.4%)	3 (33.3%)			0.71
PIs		3 (23%)	3 (33.3%)			>0.99
Metabolic abnormities						
BMI		26.1 (23.3-29.7)	32.3 (24.9-38.3)			0.14
Diabetes mellitus		1 (0.08%)	1 (0.11%)			>0.99
Hypertension		4 (30.8%)	2 (22.2%)			0.67
Abnormal lipid metabolism		5 (38.5%)	3 (33.3%)			0.67

P1: HIV- vs HIV+/αCD4^{low}

P2: HIV- vs HIV+/αCD4^{high}

P3: HIV+/αCD4^{high} vs HIV+/αCD4^{low}

Non-parametric Mann-Whitney tests.

Abnormal lipid metabolism: hyperlipidemia, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia

Multi-Class Combination ART: Two different groups in a complete HIV drug regimen (e.g., Atripla (bictegravir + tenofovir DF + emtricitabine)).

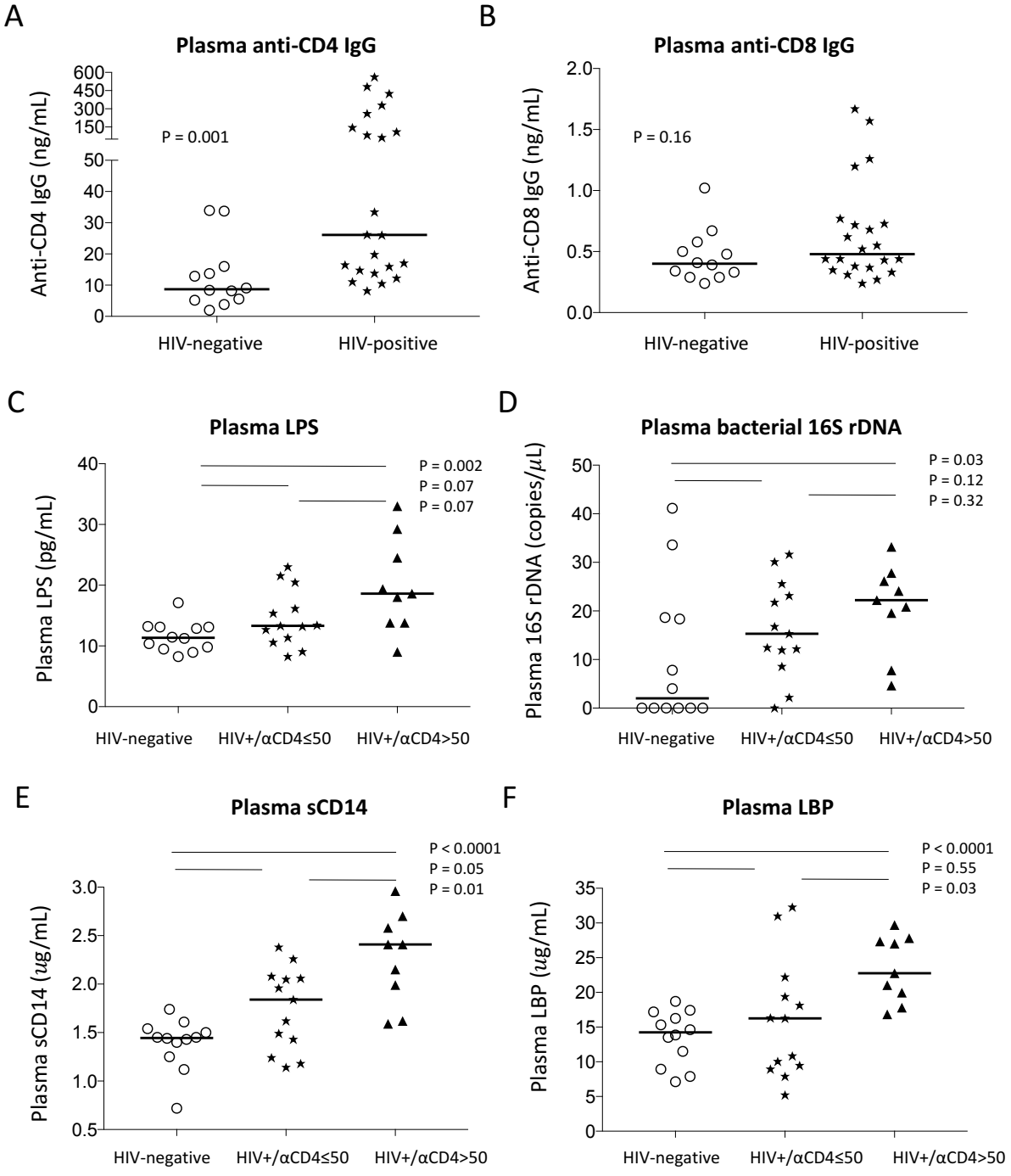

A
Plasma anti-CD4 IgG
Anti-CD4 IgG (ng/mL)
600
450
300
150
50
40
30
20
10
0
P = 0.001
HIV-negative
HIV-positive
B
Plasma anti-CD8 IgG
Anti-CD8 IgG (ng/mL)
2.0
1.5
1.0
0.5
0.0
P = 0.16
HIV-negative
HIV-positive
C
Plasma LPS
Plasma LPS (pg/mL)
40
30
20
10
0
P = 0.002
P = 0.07
P = 0.07
HIV-negative
HIV+/αCD4≤50
HIV+/αCD4>50
D
Plasma bacterial 16S rDNA
Plasma 16S rDNA (copies/μL)
50
40
30
20
10
0
P = 0.03
P = 0.12
P = 0.32
HIV-negative
HIV+/αCD4≤50
HIV+/αCD4>50
E
Plasma sCD14
Plasma sCD14 (ug/mL)
3.0
2.5
2.0
1.5
1.0
0.5
P < 0.0001
P = 0.05
P = 0.01
HIV-negative
HIV+/αCD4≤50
HIV+/αCD4>50
F
Plasma LBP
Plasma LBP (ug/mL)
35
30
25
20
15
10
5
0
P < 0.0001
P = 0.55
P = 0.03
HIV-negative
HIV+/αCD4≤50
HIV+/αCD4>50

Figure 1

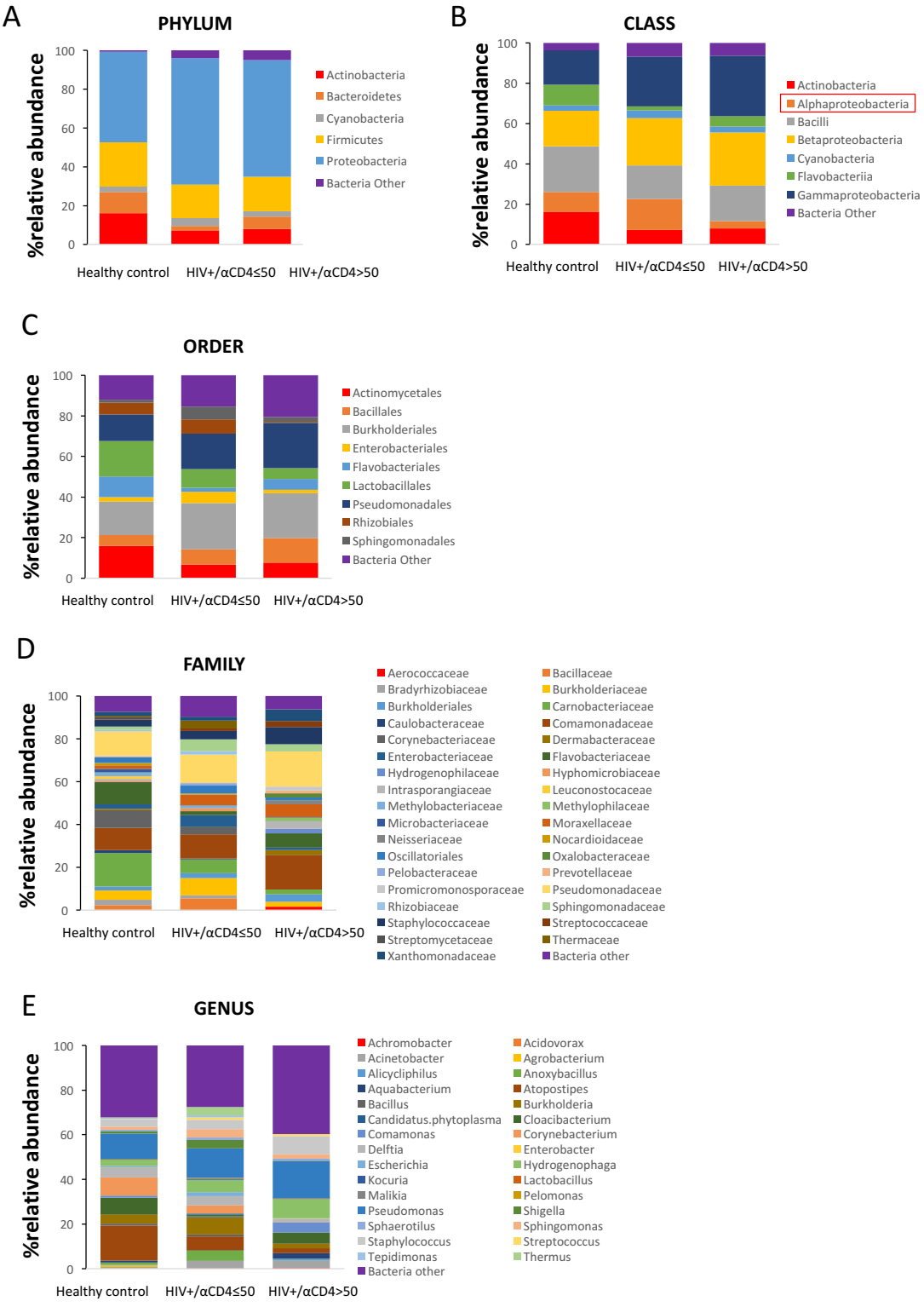

A
PHYLUM
%relative abundance
100
80
60
40
20
0
Healthy control
HIV+/αCD4≤50
HIV+/αCD4>50
Actinobacteria
Bacteroidetes
Cyanobacteria
Firmicutes
Proteobacteria
Bacteria Other
B
CLASS
%relative abundance
Healthy control
HIV+/αCD4≤50
HIV+/αCD4>50
Actinobacteria
Alphaproteobacteria
Bacilli
Betaproteobacteria
Cyanobacteria
Flavobacteriia
Gammaproteobacteria
Bacteria Other
C
ORDER
%relative abundance
Healthy control
HIV+/αCD4≤50
HIV+/αCD4>50
Actinomycetales
Bacillales
Burkholderiales
Enterobacteriales
Flavobacteriales
Lactobacillales
Pseudomonadales
Rhizobiales
Sphingomonadales
Bacteria Other
D
FAMILY
%relative abundance
Healthy control
HIV+/αCD4≤50
HIV+/αCD4>50
Aerococcaceae
Bacillaceae
Bradyrhizobiaceae
Burkholderiaceae
Burkholderiales
Carnobacteriaceae
Caulobacteraceae
Comamonadaceae
Corynebacteriaceae
Dermabacteraceae
Enterobacteriaceae
Flavobacteriaceae
Hydrogenophilaceae
Hyphomicrobiaceae
Intrasporangiaceae
Leuconostocaceae
Methylobacteriaceae
Methylophilaceae
Microbacteriaceae
Moraxellaceae
Neisseriaceae
Nocardioidaceae
Oscillatoriales
Oxalobacteraceae
Pelobacteraceae
Prevotellaceae
Promicromonosporaceae
Pseudomonadaceae
Rhizobiaceae
Sphingomonadaceae
Staphylococcaceae
Streptococcaceae
Streptomycetaceae
Thermaceae
Xanthomonadaceae
Bacteria other
E
GENUS
%relative abundance
Healthy control
HIV+/αCD4≤50
HIV+/αCD4>50
Achromobacter
Acidovorax
Acinetobacter
Agrobacterium
Alicycliphilus
Anoxybacillus
Aquabacterium
Atopostipes
Bacillus
Burkholderia
Candidatus.phytoplasma
Cloacibacterium
Comamonas
Corynebacterium
Delftia
Enterobacter
Escherichia
Hydrogenophaga
Kocuria
Lactobacillus
Malikia
Pelomonas
Pseudomonas
Shigella
Sphaerotilus
Sphingomonas
Staphylococcus
Streptococcus
Tepidimonas
Thermus
Bacteria other

Figure 2

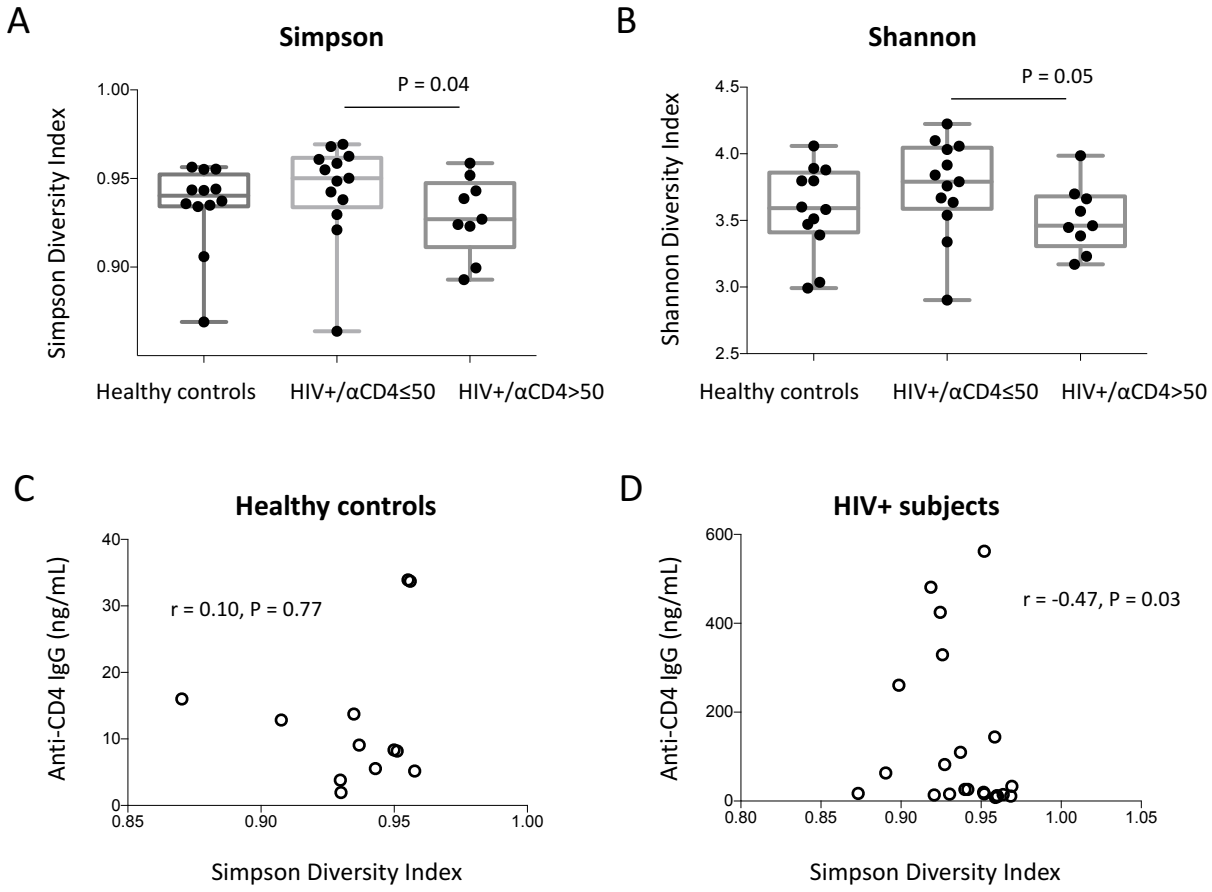
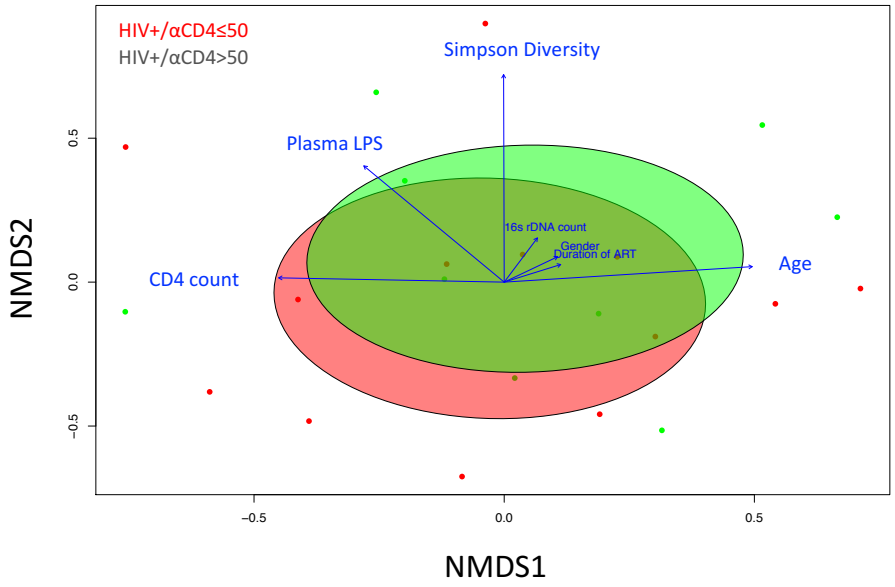

A
Simpson
P = 0.04
Simpson Diversity Index
1.00
0.95
0.90
Healthy controls
HIV+/αCD4≤50
HIV+/αCD4>50
B
Shannon
P = 0.05
Shannon Diversity Index
4.5
4.0
3.5
3.0
2.5
Healthy controls
HIV+/αCD4≤50
HIV+/αCD4>50
C
Healthy controls
r = 0.10, P = 0.77
Anti-CD4 IgG (ng/mL)
40
30
20
10
0
0.85
0.90
0.95
1.00
Simpson Diversity Index
D
HIV+ subjects
r = -0.47, P = 0.03
Anti-CD4 IgG (ng/mL)
600
400
200
0
0.80
0.85
0.90
0.95
1.00
1.05
Simpson Diversity Index

Figure 3

A


HIV+/αCD4≤50
HIV+/αCD4>50
Simpson Diversity
Plasma LPS
16s rDNA count
Gender
Duration of ART
Age
CD4 count
NMDS2
NMDS1
0.5
0.0
−0.5

B

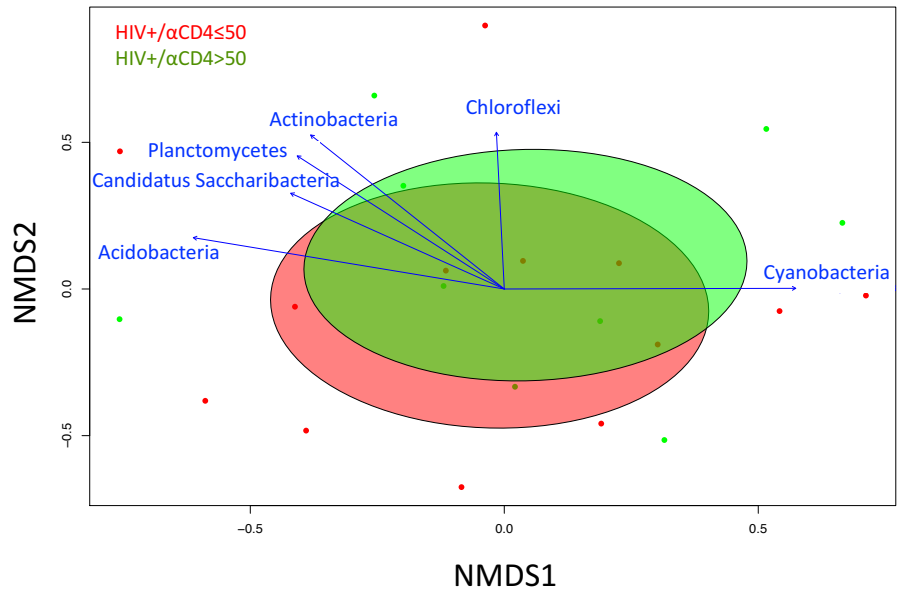

HIV+/αCD4≤50
HIV+/αCD4>50
Actinobacteria
Chloroflexi
Planctomycetes
Candidatus Saccharibacteria
Acidobacteria
Cyanobacteria
NMDS2
NMDS1
0.5
0.0
−0.5

Figure 4

CHAPTER FOUR

Running head: PAIN/STRESS AND FECAL CALPROTECTIN IN PRETERM INFANTS

The Impact of Cumulative Pain/Stress on Fecal Calprotectin Levels in Preterm Infants

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Abstract

Background*:* Preterm infants are at great risk for severe infections due to their immature immune system. However, the factors that influence the maturation of gut immune systems and their underlying mechanism remains unclear. Recent research suggests that pain/stress plays a role in immune activation and inflammatory process. Fecal calprotectin (FCP) levels have been proposed as a non-invasive biomarker measuring gut inflammation in both adults and neonates.

Objective: The purpose of the study was to examine the relationship between cumulative pain/stress and gut FCP levels in preterm infants in the neonatal intensive care unit (NICU).

Methods: The study used a longitudinal approach. Stable preterm infants were recruited in the NICU setting. Cumulative pain/stress was measured daily using a modified Neonatal Infant Stress Scale (NISS). Stool samples were collected and FCP levels were quantified using the enzyme-linked immunosorbent assay twice weekly. Both NISS scores and FCP levels were measured in preterm infants during the first four weeks of their NICU stay. Linear mixed effect model was used to examine the association among cumulative pain/stress, demographic and clinical characteristics (i.e., feeding type, antibiotics use, gestational age), and FCP levels.

Results: Thirty-one preterm infants were included in the study who were primarily White (77.4%), non-Hispanic (77.4 %), female (61.3 %), and born at 30.9 ± 1.5 weeks gestational age with premature rupture of membranes (32.3%) and by cesarean-section (77.4%). Nineteen infants (61.2%) had antibiotics during the first 48 to 72 hours post-delivery. These preterm infants experienced an average of 23.4 (± 6.3) acute painful procedures and 14.2 (± 20.3) hours of chronic painful procedures during the first four weeks of life in the NICUs. These infants' FCP levels varied largely with a mean of 267.6 ± 262.7 (μg/g) during the first four weeks of life.

Elevated FCP concentration was positively associated with chronic (prolonged) pain/stress ($p < 0.01$), antibiotics use ($p < 0.01$), and premature rupture of membranes ($p < 0.05$).

Discussion: Fecal calprotectin level was elevated in preterm infants with wide inter- and intra-individual variation. Cumulative pain/stress influences the FCP concentration in preterm infants during the first four weeks of life. These findings indicate that cumulative pain/stress is positively associated with the gut inflammatory process.

Key Words: Fecal calprotectin, gut inflammation, preterm infant, pain, stress, ELISA

The Impact of Cumulative Pain/Stress on Fecal Calprotectin Levels in Preterm Infants

Approximately 10% of infants are born prematurely every year in the U.S (Hamilton, Martin, Osterman, Curtin, & Matthews, 2015). Preterm infants are vulnerable to morbidity and mortality related to severe infections (i.e., neonatal sepsis, necrotizing enterocolitis). The mortality rate of preterm infants with systemic infection is up to 40 percent in the United States (McGuire, Clerihew, & Fowlie, 2004). The increased risks for infection in preterm infants is profoundly due to the developmental immaturity of the gut, which characterized by immature (leaky) mucosal and epithelial barriers, increased intestinal permeability as well as an immature immune system (Sharma, Jen, Butler, & Lavoie, 2012).

The factors that influence the maturation of gut immune systems are complex and multifactorial. Preterm infants are exposed to numerous stressors (medical, psychosocial and environmental) in the Neonatal Intensive Care Unit (NICU). Evidence shows that increased exposure to stressors in early life stimulates pro-inflammatory compounds and leads to long-term immune cell activation (Grunau, 2013). However, the underlying mechanism of early life stress altering immune functions remains unclear. An expanding area of research supports that the function of the brain-gut axis plays a key role in the regulation of stress and early programming of the neuro-immune system (Cong, Henderson, Graf, & McGrath, 2015; Mayer, Tillisch, & Gupta, 2015). Physical and emotional stress, including painful procedures and maternal separation, may result in altered epithelial barrier integrity, increased intestinal permeability (leaky gut), up-regulation pro-inflammatory mediators, and activation of immune and somatic cells through pathogen-associated receptors that trigger inflammation (Ghaisas, Maher, & Kanthasamy, 2016; M. W. Groer, Gregory, Louis-Jacques, Thibeau, & Walker, 2015; M. W. Groer et al., 2014; Moloney, Desbonnet, Clarke, Dinan, & Cryan, 2014).

Fecal calprotectin (FCP) is a non-invasive biomarker that measures inflammation in the GI tract (M. Groer, Ashmeade, Louis-Jacques, Beckstead, & Ji, 2016; Ho et al., 2015; Kawashima et al., 2016; van Rheenen, Van de Vijver, & Fidler, 2010; Walsham & Sherwood, 2016). Fecal calprotectin is a calcium-binding protein that is primarily excreted by granulocytes, and also expressed by monocytes/macrophages and epithelial cells. FCP accounts for about 60% of the cytosolic proteins in neutrophil granulocytes (Dale, Brandtzaeg, Fagerhol, & Scott, 1985) and is involved in intracellular signal transduction, neutrophil defense, immunoglobulin production and regulating inflammatory responses (Roseth, Fagerhol, Aadland, & Schjonsby, 1992). Elevated FCP has been shown to be related to the migration of neutrophil granulocytes into the intestinal mucosa toward the lumen, which usually occurs during intestinal inflammation (Bjerke, Halstensen, Jahnsen, Pulford, & Brandtzaeg, 1993; Roseth, Schmidt, & Fagerhol, 1999). Moreover, FCP has been successfully used in detecting inflammation among patients with inflammatory bowel disease (IBD) (Kawashima et al., 2016; van Rheenen et al., 2010; Walsham & Sherwood, 2016) and necrotizing enterocolitis patients (Albanna, Ahmed, & Awad, 2014; Aydemir et al., 2012; Benitez & Garcia-Sanchez, 2015; Yoon et al., 2014). Calprotectin has been proposed as a biomarker predicting IBD relapse, with a sensitivity of 90% and a specificity of 83% (Tibble, Sigthorsson, Bridger, Fagerhol, & Bjarnason, 2000). As an extremely stable protein, FCP has become a newer diagnostic tool to detect intestinal inflammation.

The purpose of the study is threefold: 1). To describe the FCP levels in preterm infants during the first four weeks of life; 2). To examine the association between cumulative stress in early life and FCP levels among preterm infants; 3). To explore the contributing factors that are associated with FCP levels.

Methods

Study Design and Participants

This study used a secondary analysis approach. The original data were collected by Cong et al., in relation to increasing understanding of the gut/brain axis and the microbiome (#1K23NR014674). From October 2013 through June 2017, a total of 93 infants have been recruited from the Connecticut Children's Medical Center (CCMC) NICUs at Hartford and Farmington, CT.

The original study used a prospective longitudinal design to examine preterm infants' gut microbiome patterns over the first four weeks of NICU stay. Infants were followed in the NICU for 3-4 weeks after birth and information on cumulative pain/stress, medication use and feeding types were collected daily during the study period. Stool samples were also collect daily from birth till 28 days of postnatal age. A neurodevelopment assessment was performed at an average of 38 weeks of corrected gestational age to examine long-term outcomes. The demographic characteristics of the infants of the original study included healthy infants born between 26–32 weeks gestational age, male (52%), White (73%), non-Hispanic (71%), and Cesarean-section births (67%) (Cong, Judge, et al., 2017).

Setting and Sampling

Infants from the original study who meet the eligibility criteria were randomly selected for this secondary analysis. **Inclusion criteri**a: Infants who were 26–32 weeks gestational age at birth, aged 0–3 days old and cared for in an incubator. The infant's mother and father had to be ≥ 18 years old to give consent. **Exclusion criteria**: Infants who had: 1) known congenital anomalies; 2) severe periventricular/intraventricular hemorrhage (≥ Grade III); 3) undergone minor or major surgery including procedures such as inguinal hernia repair, laparotomy,

thoracotomy, diaphragmatic hernia repair, or intestinal resection; and 4) history of illicit drug exposure during the current pregnancy. We randomly select 31 infants from the original study who had complete daily pain/stressor data and also had at least one stool sample per week for the first four weeks.

Outcome Measures and Data Collection

Demographic data and health characteristics. Demographic information including gestational age at birth, birth weight, sex, mode of delivery, the timing of rupture of membranes was extracted from the medical record. The severity of illness soon after birth was measured by the Score for Neonatal Acute Physiology–Perinatal Extension II (SNPPEII) (Richardson, Corcoran, Escobar, & Lee, 2001). Information on postnatal antibiotics administration including generic names and duration of antibiotics use (day) were also collected.

Cumulative Pain/stress. A modified version of the Neonatal Infant Stressor Scale (NISS) (Newnham, Inder, & Milgrom, 2009) was used to assess daily cumulative pain and stress (Cong, Wu, et al., 2017). The modified NISS instrument consists of 70 acute and chronic painful/stressful procedures and events that are commonly experienced by neonates in the NICU including daily care, feeding, imaging, blood draw, peripheral venous access, central venous access, respiratory care, surgeries and major procedures, and infection. Each procedure/event is assigned to a pain severity level from 1 to 5 (1= not painful/stressful; 2 = a little; 3 = moderate; 4 = very; and 5 = extremely painful/stressful). Acute stress scores are compiled by summing up the weighted frequency of each acute event, whereas chronic stress scores are compiled by summing up the weighted duration of each chronic event. Cumulative pain and stress composite scores were calculated daily based on daily pain/stressor data for the first 4 weeks of life in preterm infants.

Fecal Calprotectin Assay. Daily stool samples from preterm infants were collected by research nurses in the original study using sterile, disposable spatulas during diaper changes and then placed into a sterile specimen container. Samples were immediately frozen upon collection at -80°C, then transferred to the lab and stored at -80°C until processing. The stool samples on the third and seventh day of each week from each infant were selected for the FCP assay and analysis. A hundred milligram stool from each stool sample was aliquoted to a 15 ml falcon tube for FCP ELISA assay using the PhiCal kit (Calprest®, Eurospital S.p.A, Trieste, Italy) according to the manufacturer's instructions. All the samples and standard controls from the kits were tested in duplicate. The inter-assay and intra-assay coefficients of variation were calculated for quality control. The mean coefficient of variation was less than 3% (Rouge et al., 2010). The reported sensitivity and specificity of the PhiCal test in pediatric population were 93.2% and 86.6% respectively (Radillo, Pascolo, Martelossi, Dal Bo, & Ventura, 2016).

Feeding Type. Infant daily feeding information including the frequency of infant fed by mother's milk (MOM), human donor's milk (HDM), and formula over the first four weeks of life was also extracted from the medical record. The frequency and percentage of each feeding type (MOM, HDM, or formula) for each infant were calculated daily and over each week. We also categorized study infants into MOM group and non-MOM groups based on >70% of the total frequency of feeding of the first four weeks. For example, an infant fed MOM for more than 70% of the total feeding time was assigned to the MOM group.

Statistical Analysis

Data were analyzed using the R (version 3.2.4) software packages. Descriptive methods were used to generate summary statistics of study variables. Effects of acute and chronic pain/stress on FCP levels were assessed via a general linear mixed-effects regression modeling

using the lme4 package in R (Bates, Mächler, Bolker, & Walker, 2015). Missing data were deleted from the analysis. We obtained p values for regression coefficients using the car package (Fox & Weisberg, 2011). Variance inflation factors were performed using R to evaluate the severity of multicollinearity among the factors of mixed effect model. GGplot2 package in R was used to generate figures to demonstrate the relationship between cumulative pain/stress and FCP levels.

Results

Participants

Thirty-one preterm infants were randomly selected for inclusion in this study from the larger study sample. The demographic characteristics were indicated in Table 1. A total of 194 stool samples were aliquoted for FCP analysis with an average of 5.5 (± 1.4) stool samples from each infant over the 4-weeks period after birth. The earliest stool sample that was obtained for FCP analysis was on the postnatal day 3.

Fecal Calprotectin concentration

The mean value and standard deviation (SD) of FCP levels were 267.6 ± 262.7μg/g, with a wide range of 23.1 μg/g to 1320.2 μg/g. The inter- and intra-individual coefficients of variation were 81.4% and 76.86% respectively. The FCP levels decreased significantly from week 1 (mean ± SD: 309.2 ± 287.2 μg/g) to week 2 (mean ± SD: 160.0 ± 201.3 μg/g) and then increase on week 3 (mean ± SD: 272.6 ± 260.1 μg/g) and week 4 (mean ± SD: 351.7 ± 290.6 μg/g) (Figure 1). One-way ANOVA of mean FCP levels significantly differed among 4 weeks ($F(3,190) = 5.609$, $p = 0.001$). Tukey's honest significant difference (HSD) post hoc test indicated that mean FCP levels were significantly different between week1 and week 2, and week 2 and week 4.

Cumulative pain/stress and relations with FCP values

Preterm infants experienced an average of 23.4 (± 6.3) acute painful procedures daily and 14.2 (± 20.3) hours of daily chronic painful procedures during the first four weeks of life in the NICU. Figure 2 shows the average unweighted amount of weekly acute (2A) and chronic (2B) pain/stressors during the first, second, third, and fourth week. Linear regression model showed a decreased trend in both the daily acute NISS scores ($F(1,182) = 26.06$, $p < 0.001$, $R^2 = 0.03$), and daily chronic NISS scores ($F(1,182) = 49.28$, $p < 0.001$, $R^2 = 0.06$) over the first four weeks of infant life in the NICU. In addition, both acute pain/stress ($F(4, 182) = 6.059$, $p < 0.05$) and chronic pain/stress ($F(4, 182) = 6.571$, $p < 0.05$) were positively associated with the FCP level after controlling post-natal weeks (Figure 3).

Feeding types and relations with FCP values

The initial enteral feedings were introduced between postnatal Day 1 to Day 6 (Mean = 2.8, SD = 1.3). The feeding regimens for preterm infants were mixed with mother's milk (MOM), human donor milk (HDM) and formula, except two infants who were fed exclusively with formula over the 4-week period. The overall percentage of feeding type for each infant during the first four weeks of life included 50.9% (± 29.0) of MOM, 25.4% (± 26.4) of HDM, and 23.8% (± 28.3) of formula. Nothing by mouth (NPO) status during the first four weeks of life ranged from 0 to 5 days (M=1.4, SD = 1.3). Infants with a higher percentage of MOM ($F(4,168) = 6.8$, $p = 0.057$ $R^2 = 0.12$) and formula ($F(4,168)=0.04$, $p = 0.32$, $R^2 = 0.10$) tended to have an increased FCP level across time (Figure 4). In contrast, infants with a higher percentage of HDM ($F(4,168) = 9.6$, $p < 0.001$, $R^2=0.17$) tended to have a lower FCP level over the four week period.

Antibiotics use and relations with FCP values

Nineteen infants had antibiotics (Ampicillin and Gentamicin) during the first 48 to 72 hours post-delivery, among those three infants had antibiotics for 10 days or above. There was no difference in the FCP level in infants who used antibiotics compared to infants without antibiotics use during the first three weeks ($p > 0.05$). However, the FCP level became significantly higher in infants who used antibiotics (433.4 ± 302.3 μg/g) during week 4 compared to infants without antibiotics use (147.4 ± 100.7 μg/g) ($t(39.3) = -4.58$, $p < 0.001$) (Figure 5).

Mixed-effects model

To determine the effect of chronic pain/stress and other contributing factors to the FCP level, a general linear mixed effects model was conducted with FCP concentration as an outcome measure and pain/stress, feeding types, antibiotics use and demographic variables as the predict factors. The log transformation was applied to the FCP level to obtain a normal distribution. The analysis of the mixed effects model showed that an increased FCP level was associated with higher daily chronic pain/stress ($p < 0.01$), increased percentage of HDM intake ($p < 0.01$), premature rupture of membranes at birth ($p < 0.01$) and postnatal antibiotic therapy ($p < 0.05$). Factors including gestational age at birth, sex and mode of delivery were not significant (Table 2). No evidence of multicollinearity was found given that all the variance inflation factors (VIF) were ≤ 5. A separate mixed effects model of acute pain/stress and FCP level shows similar results on the contributing factors described above, including feeding type, premature rupture of membranes at birth, postnatal antibiotic therapy, gestational age at birth, sex and mode of delivery, however, the effect of acute pain/stress disappeared ($p = 0.064$).

Discussion

The current study showed that FCP levels of preterm infants were elevated compared to a healthy standard. Previous studies also reported that FCP levels were higher in preterm infants compared to full-term cohorts (Kapel et al., 2010; Rouge et al., 2010). High inter-individual and intra-individual coefficients of variability were also observed in this study. Prior to proceeding with further data analysis and interpretation, technique and procedures of FCP assay were reviewed to account for any technical errors of measurement. All the samples and standard controls were tested in duplicate with low inter-assay and intra-assay coefficient of variation; both were lower than 10%, thus eliminating the error from the ELISA assay. Another concern arises with the stool collection technique, where water is absorbed into the diaper which leads to increased FCP level-up to 30% (Olafsdottir, Aksnes, Fluge, & Berstad, 2002). However, the variation observed in our study far exceed the variation caused by water absorption. In addition, based on the protocol of the study site, the diaper was changed every two hours. Once the sample was collected from the diaper, it was immediately transferred to a -80ºC freezer until processing. It is unlikely that FCP degraded during the data collection procedure. Therefore, we concluded that our data reflected true high concentration and variability of FCP in preterm infants. Our study findings were consistent with previous studies showing high FCP levels and large inter- and intra-individual variation in preterm infants (Josefsson, Bunn, & Domellof, 2007; Moussa, Khashana, Kamel, & Elsharqawy, 2016; Rouge et al., 2010).

Fecal calprotectin, a cytoplasmic protein in neutrophil granulocytes is primarily found in the gut when the neutrophils migrate to the intestinal mucosa. Since calprotectin is not present in human's milk (Olafsdottir et al., 2002), a high FCP level should be closely associated with the gut inflammation of preterm infants. One of the explanation is that preterm infants are more

likely to have immature intestinal mucosal and enhanced intestinal permeability (Weaver, Laker, & Nelson, 1984), which leads to increased trans-epithelial migration of neutrophils through intercellular junctions, and release calprotectin into the gut lumen (Berstad, Arslan, & Folvik, 2000; Parkos, Colgan, Delp, Arnaout, & Madara, 1992). Our study found no correlation between the FCP level and gestational age before 34 weeks. It is possible that the level of FCP before 34 weeks remains constantly high and does not vary significantly within the age window. Our findings add more specificity to the previous studies of the FCP level and gestational age (Campeotto et al., 2007; Josefsson et al., 2007; Li et al., 2014; Moussa et al., 2016; Yang, Smith, Goldberg, & Cotten, 2008).

Meanwhile, controversial relationships between the FCP level and postnatal age have been reported previously (Josefsson et al., 2007; Li et al., 2014). We found that FCP levels decreased from the first to the second week, and then increased till the 4th week of life. This finding agrees with Josefsson's (2007) study that the FCP level decreases during the first week postnatal and then increase to the 8th week. The elevated FCP level in meconium may not reflect the gut inflammation during the first week of life but due to the cumulative effect of FCP in meconium during pregnancy.

The FCP level was significantly influenced by feeding regimen across time. Study infants who received a higher percentage of MOM feeding tended to have an increased FCP level across time. However, the increase was marginally significant, probably due to small sample sizes. In contrast, infants who were fed with a higher percentage of HDM or formula tended to have a lower FCP level. Several studies have investigated the relationship between feeding regimen and FCP and reported inconsistent findings. Our findings are in line with Groer's study (2015) that exclusive MOM was found to be associated with an elevated FCP level in very low birth weight

infants during the first four weeks of life. Li (2014) and Asgarshirazi (2017) also reported the FCP level is higher in breastmilk-fed infants than non-breast fed ones. In contrast, Rouge (2010) found that the FCP level was significantly elevated in preterm infants who received exclusive or predominant formulas. Other authors found that feeding regimen was not significantly correlated with FCP (Campeotto et al., 2007; Josefsson et al., 2007; Yang et al., 2008).

The increased FCP level may reflect protective mechanisms of MOM to the immature gut and represent the maturation of gut immunity. MOM contains microbiota and oligosaccharides, which are essential in the establishment of commensal bacteria, and promoting the maturation of preterm infants' gut. In our previous study, we reported that MOM promotes the diversity of gut microbiome and early transition to adult-like microbial patterns in preterm infants compared to HDM and Formula (Cong, Judge, et al., 2017; Xu et al., 2017). Rouge's (2010) study confirmed the effect of enteral bacterial in promoting FCP production, which could explain the postnatal changes of the FCP level in our population. In addition, MOM contains more than 10^9 leukocytes per liter for the first several months of lactation as well as other bioactive substances, such as pro-inflammatory cytokines, antibodies and other immune-stimulating factors (Asgarshirazi, Shariat, Nayeri, Dalili, & Abdollahi, 2017; Newburg & Walker, 2007; Wallace et al., 1997). Thus, the elevated level of FCP could also be a response to these immune factors present in MOM. Infant fed predominately with HDM showed a lower level of FCP concentration, which may due to the pasteurization removed commensal bacteria and inactivate immune components (Cong, Judge, et al., 2017; Ewaschuk, Unger, Harvey, O'Connor, & Field, 2011; Xu et al., 2017). Nineteen of the 31 infants received antibiotics postnatally, predominately Ampicillin and Gentamycin during the first 48 to 72 hours. There were only three infants in this study received extended antibiotics after the first week. Interestingly, we observed that FCP levels were similar

in infants with or without antibiotics use during the first three weeks, but then increased to a significantly higher level during the 4th week in infants who received postnatal antibiotics compared to infants who did not use antibiotics. However, Josefsson (2007) reported that FCP levels were significantly lower in infants with postnatal antibiotics use, specifically use of Cefotaxime and Meropenem compared to infants without antibiotic therapy. Since the types of antibiotics used in Josefsson's study were different from the antibiotics in our study, therefore, results were not comparable. Rouge (2010) also reported a lower FCP level in infants with ante- and post-natal antibiotic use, but the types of antibiotics were not reported in the study. Groer (2015) found no association between FCP and antibiotics use in a study that the majority of infants were given postnatal antibiotics, possibly due to the homogeneity of the antibiotics use. Antibiotics have been known to influence gut bacterial colonization and disrupt the microbial community. We speculate that prophylactic antibiotics use during the first 48-72 hours disrupt the infant's gut microbiota colonization, particularly commensal bacteria during the first week of life. The imbalanced of gut community leads to a growth of certain groups of bacteria, which corresponded to an elevated FCP later during the first month. This speculation was also supported by findings in our study that infants with premature rupture of membranes have elevated FCP value.

We found that both acute and chronic daily pain/stress are significantly positively correlated with the FCP level. However, when using a mixed-effect model and controlling for other variables, only the effect of chronic pain/stress on FCP remained significant. The effect of acute pain/stress on FCP became marginal significant, which may be due to small sample sizes. This study is the first to report the relationship of cumulative pain/stress experience and the FCP level in preterm infants. The findings supported our hypothesis that stress may alter epithelial

barrier integrity, increase intestinal permeability, leading to activation of the inflammatory process in the gut. Notably, the NISS scores were calculated daily based on the number of medical procedures the infants experienced in the NICUs. Therefore, FCP may also reflect the inflammation that was directly caused by these medical procedures, as well as the infants' medical conditions.

There are several limitations to this study. First, the mother's information was not collected in this project. Mother's antenatal conditions, such as medical complications, antibiotics use may influence the FCP level, especially during the first week. Common conditions like intra-uterine inflammation, causes premature immune activation and cytokine production and may account for the elevated FCP level in preterm infants. Secondly, we did not include measures of other inflammatory markers such as cytokines (e.g., interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor (TNF)), c-reactive protein or cortisol in this study to better understand the sources and causes of the inflammation. Therefore, the effect of FCP as a marker in the prediction of infants' health outcome is still inconclusive. Thirdly, gut microbiome data have not been included in this analysis to examine the correlations between microbiome, FCP and stress. However, our study results suggest that gut microbiome may be an essential pathway in modulating the effect of external stressors on the gut inflammatory process. Further studies need to be done exploring the mediation effect of gut microbiome patterns (i.e., diversity, bacterial composition and abundance) on external stimuli, particularly stress, on gut inflammation.

Conclusion

This study demonstrates that preterm infants have high calprotectin concentrations with wide inter- and intra-individual variation during the first four weeks of life. The elevated FCP level was positively associated with MOM intakes, postnatal antibiotics use during the first 72 hours, pre-rupture of membranes and prolonged pain/stress during NICUs staying. Further work should examine the association and mechanisms of FCP influences health in preterm infants.

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Table 1. Demographic and clinical characteristics of the preterm infants (N=31)

Demographic		n	Percent (%)
Gender	Male	12	38.7
	Female	19	61.3
Race	White	24	77.4
	African American	6	19.4
	Not known	1	3.2
Ethnicity	Hispanic	7	22.6
	Non-Hispanic	24	77.4
Delivery type	Vaginal	7	22.6
	Cesarean section	24	77.4
PROM	Yes	10	32.3
	No	21	66.7
Birth	Multiple birth	11	37.9
	Single birth	17	62.1
Resuscitation at birth	Yes	24	77.4
	No	7	22.6
Antibiotic Use	First 48 hours	19	61.2

	Mean (SD)	**Range**
Gestational age (wks)	30.9 (1.5)	27.9 - 33.4
Birth weight (g)	1505.3 (445.7)	703 - 2640
Birth length (cm)	40.5 (3.6)	32.5 - 47
Birth head circumference (cm)	28.3 (2.1)	24.0 - 34.5
SNAPEII	7.9 (7.8)	0 - 29
Mother age (yrs)	30.7 (8.3)	19 - 46
Antibiotic use total (days)	2.6 (3.5)	0 -15

Table 2 Mixed Effects Model: Factors contributing to fecal calprotectin level

	Estimate	SE	t-value	p	VIF
Time interval					
Week 2	-0.585	1.798	-0.325	0.745	1.502
Week 3	-0.123	1.798	-0.069	0.945	1.502
Week 4	0.208	1.801	0.115	0.908	1.502
Chronic pain/stress	0.042	0.016	2.628	0.009**	1.072
MBM (%)	-0.189	0.276	-0.684	0.494	1.357
HDM (%)	-1.308	0.484	-2.702	0.007**	1.284
Pre-rupture of membrane	0.670	0.210	3.198	0.001**	1.110
Postnatal antibiotics	0.494	0.249	1.981	0.048*	1.551
Birth Gestational age	-0.043	0.080	-0.542	0.588	1.535
Sex	0.239	0.209	1.146	0.252	1.151
Delivery mode	0.225	0.305	0.740	0.459	1.873

Note: N=29; Log transformation was performed to fecal calprotectin Level.

Figure Captions:

FIGURE 1. Fecal calprotectins in preterm infants during the first 4 weeks of life. The boxplot shows the median (horizontal line) and includes the 25th (lower box border) to 75th (upper box border). One-way ANOVA of mean FC levels are significantly differs among 4 weeks ($F(3,190) = 5.609$, $p = 0.001$).Tukey's honestly significant difference (HSD) post hoc test indicate mean FC level is significantly different between week1 and week2; week 2 and week4.

FIGURE 2. Unweighted weekly mean pain/stressors during the first 4 postnatal weeks decomposed by pain/stress severity levels. 1A - Acute pain/stressors (frequency); 1B - Chronic pain/stressors (hours).

FIGURE 3. Fecal calprotectin levels in preterm infants who had A. low (blue) and high (red) acute B. low (blue) and high (red) chronic pain/stress procedure scores during first four postnatal weeks. The dot and the error bar represent the mean value and standard error of the FCP in each week.

FIGURE 4. Fecal calprotectin levels in preterm infants who were fed with MOM and non-MOM during first four postnatal weeks. The dot and the error bar represent the mean value and standard error of the FCP in each week. The red line represents infants who were fed with primarily with MOM for more than 70% of the entire feeding. The blue line represents infants who were fed with primarily with non-MOM for more than 70% of the entire feeding. MOM= mother's own milk; non-MOM= non-mother's own milk.

FIGURE 5. Fecal calprotectin levels in preterm infants with and without antibiotics use during first 4 postnatal weeks. The dot and the error bar represent the mean value and standard error of the FCP in each week. The red line represents infants who had antibiotics use during the first 48-72 hours of life. The blue line represents infants without antibiotics use.

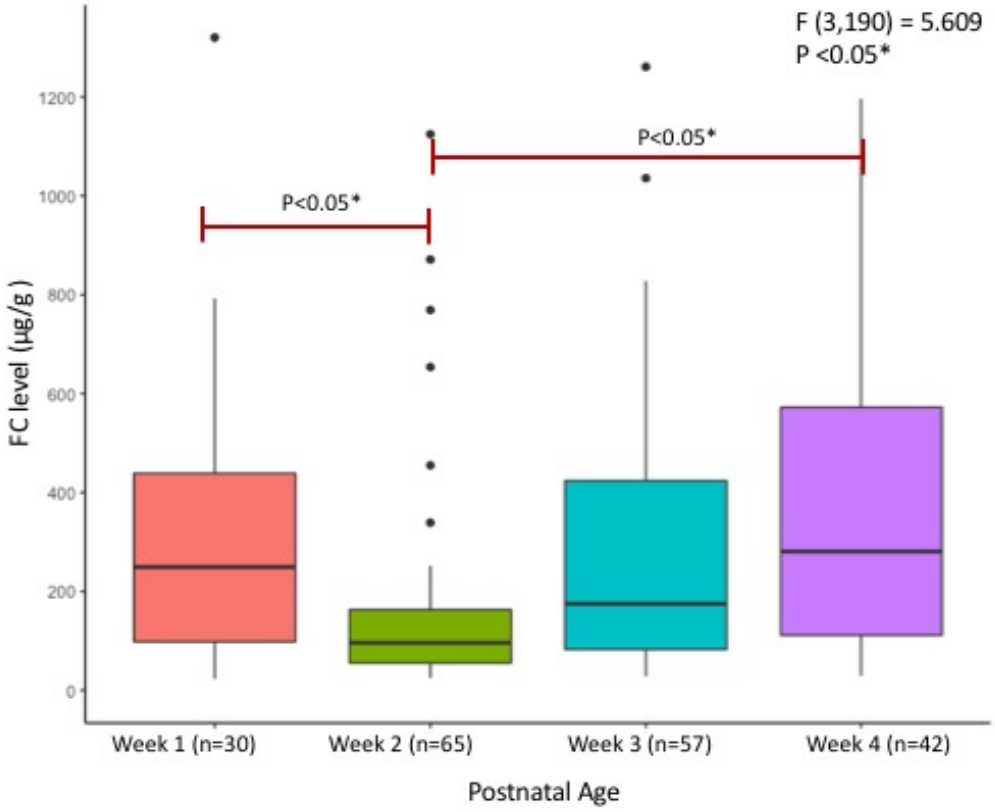
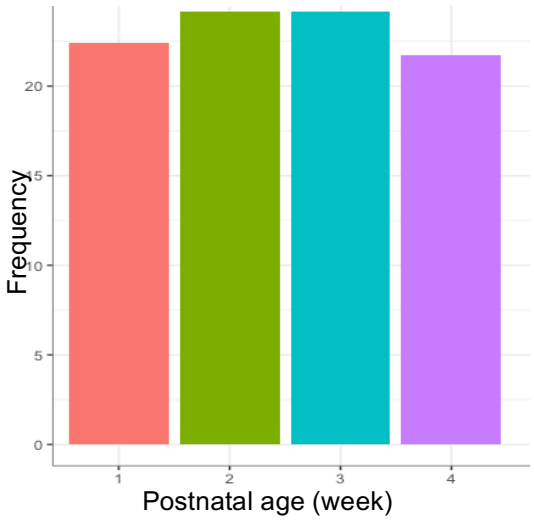

F (3,190) = 5.609
P <0.05*
P<0.05*
P<0.05*
FC level (µg/g)
0
200
400
600
800
1000
1200
Week 1 (n=30)
Week 2 (n=65)
Week 3 (n=57)
Week 4 (n=42)
Postnatal Age

Figure 1

A. Acute painful/stressful procedures



B. Chronic painful/stressful procedures

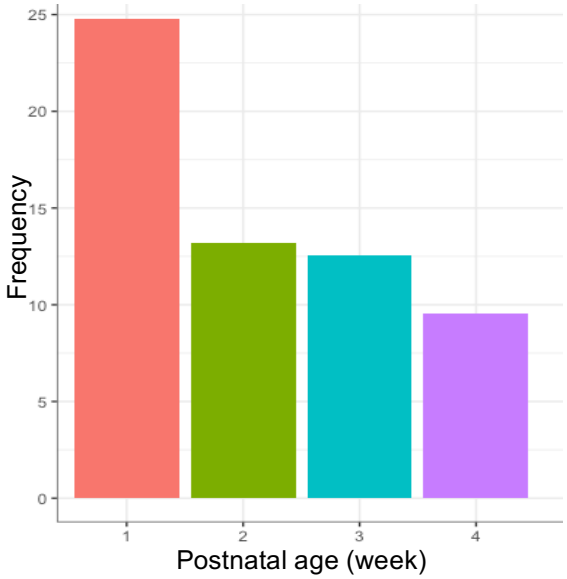
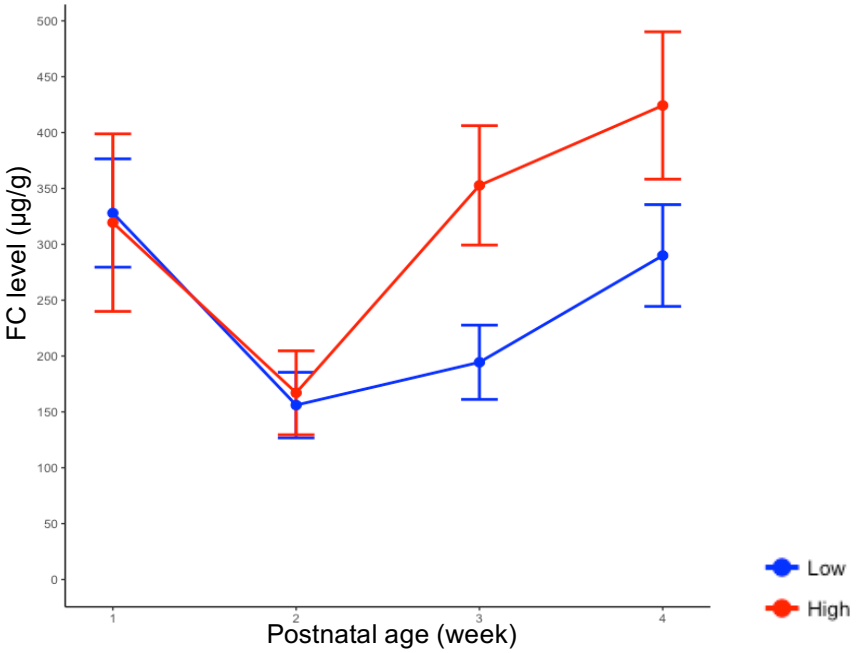


Figure 2

A. Acute



B.

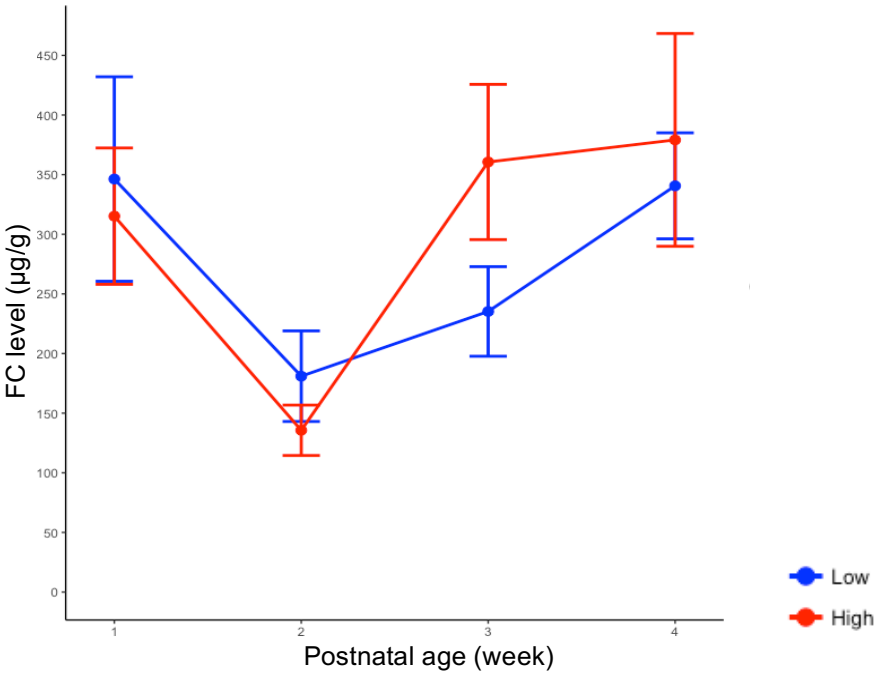


Figure 3

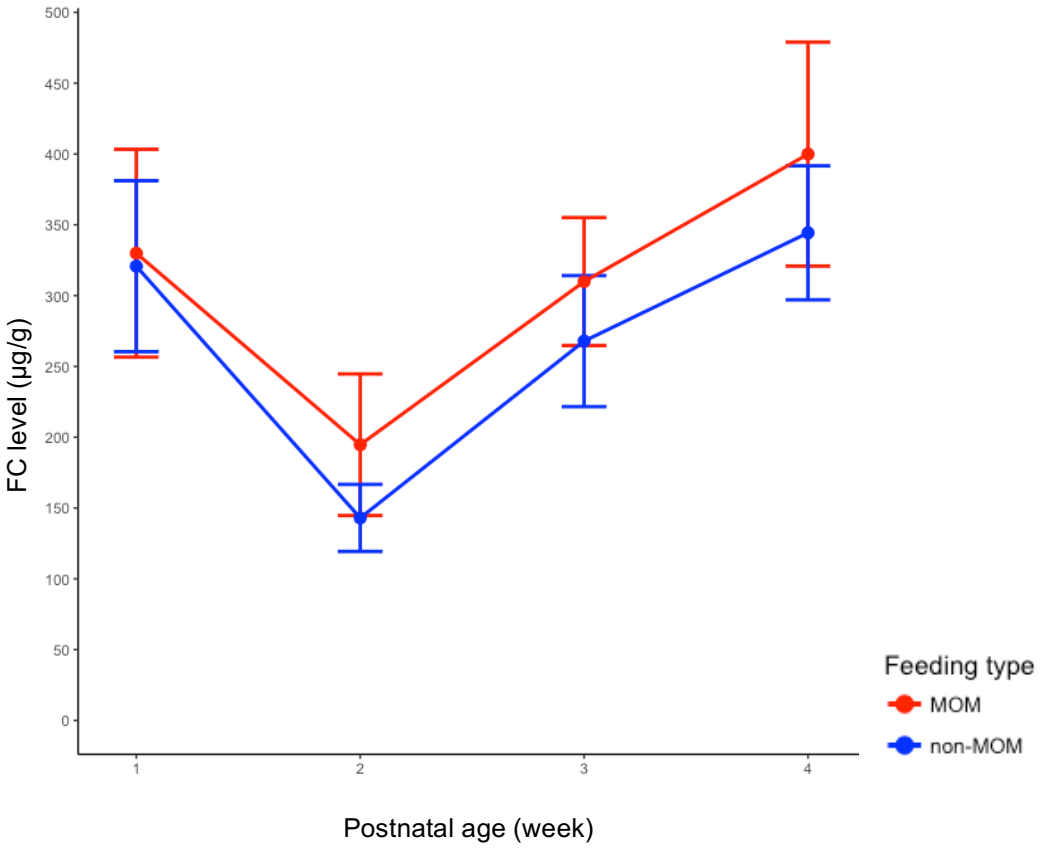

500
450
400
350
300
250
200
150
100
50
0
FC level (µg/g)
1
2
3
4
Postnatal age (week)
Feeding type
MOM
non-MOM

Figure 4

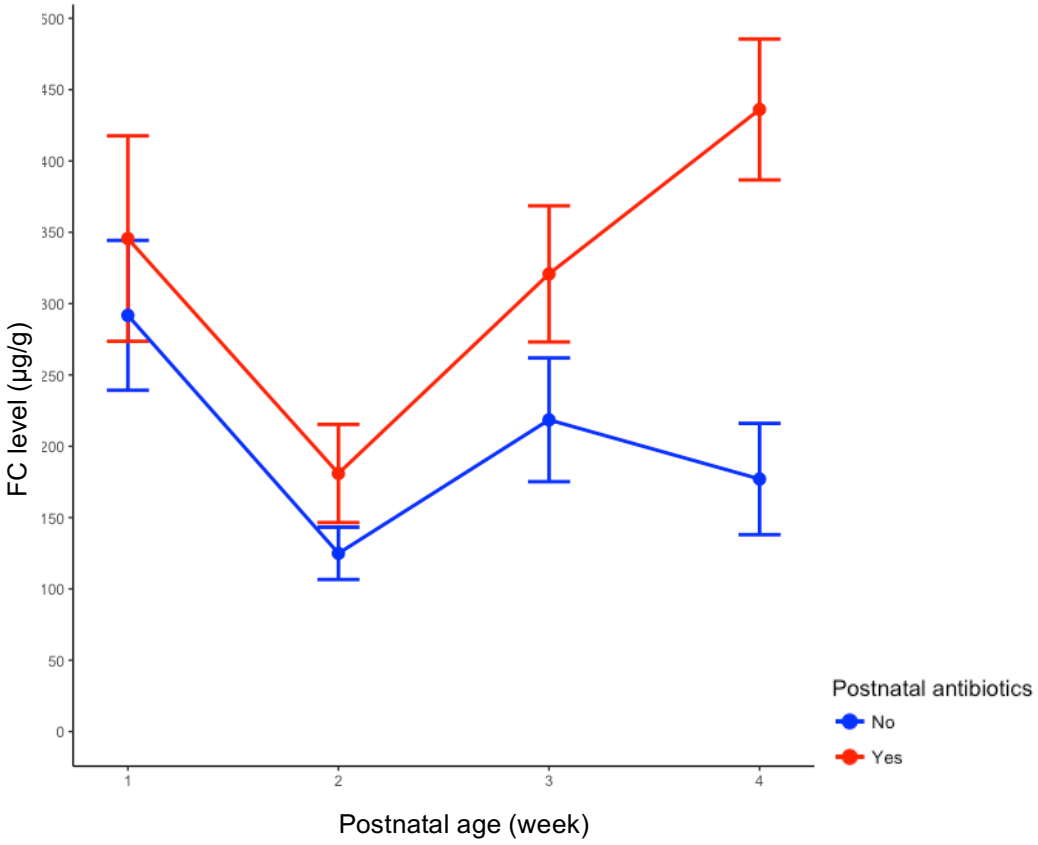

500
450
400
350
300
250
200
150
100
50
0
FC level (µg/g)
1
2
3
4
Postnatal age (week)
Postnatal antibiotics
No
Yes

Figure 5

CHAPTER FIVE

5.1 Introduction

This dissertation focuses on the interplay between pain/stress, inflammation and microbiota in human health. Three studies are reported herein. The study reported in chapter 2 is the development and testing of a tool to assess cumulative pain/stress in early life, a critical factor that affects the HPA axis of brain-gut-microbiota pathway. The study reported in chapter 3 examined the effect of gut microbiome translocation on adaptive immune responses in patients with immunodeficiency. Chapter 4 provides the results of a study that examined the effect of cumulative pain/stress on gut inflammation and early programming the innate immune system. Cumulatively the results of the dissertation fill several gaps in our knowledge related to modulation of immune responses in infants and HIV patients. Overall the results demonstrate that the brain-gut-microbiota axis plays an important role in the modulation of both innate and adaptive immune responses.

5.2 Major findings from Chapter 2

Exposure to prolonged and cumulative stressors in early life can impair the early programming of neuro-immune system, particular in the vulnerable neonates who are born prematurely (Anand, Palmer, & Papanicolaou, 2013; Anand & Scalzo, 2000; Brummelte et al., 2012; Fitzgerald & Beggs, 2001; Ranger & Grunau, 2014). However, most existing assessment tools have been developed to measure acute pain over a short period; few valid instruments are available to measure cumulative pain/stress among NICU infants over time.

A nationwide online survey was conducted through the U.S. National Association of Neonatal Nurse's membership to assess nurses' perceptions about severity and acuity levels regarding 68 painful/stressful procedures that infants commonly experience in the NICU. Based on the nurses' responses, each procedure was classified as acute, chronic, or both, and was

assigned a severity level from a 5-point Likert scale, with one indicating not painful/stressful and five indicating extremely painful/stressful. These results provide preliminary data for the development of an objective tool to measure cumulative pain/stress that may be generalized nationally with additional testing. Upon further validation, this tool will become more precise in measuring prolonged infant pain longitudinally.

5.3 Major findings from Chapter 3

Chronic inflammation has been a critical issue in HIV disease even in patients under viral suppressive antiretroviral therapy (ART). The underlying mechanisms of long-term humoral immune perturbations remain largely unknown. The study in Chapter 3 investigated the association between microbial translocation, and plasma anti-CD4 autoantibodies in HIV+ subjects under long-term viral suppressive ART treatment.

The study found that plasma level of anti-CD4 IgGs but not anti-CD8 IgGs was increased in HIV+ subjects compared to healthy controls. Importantly, plasma microbial patterns differed between healthy controls and HIV-infected patients and between patients with low, and high plasma anti-CD4 IgG production. Increased plasma anti-CD4 autoantibodies production in HIV-infected individuals was found to be associated with reduced plasma microbial diversity and a reduced level of *Alphaproteobacteria.* These results suggest that elevated systemic microbiota may play a role in the induction of anti-CD4 autoantibodies in HIV infection. The study provides evidence which supports systemic microbiota and microbial translocation from the gastrointestinal tract to systemic circulation as a major driver of persistent systemic inflammation in HIV disease.

5.4 Major findings from Chapter 4

A secondary analysis of a longitudinal observational data was conducted to examine the

impact of cumulative pain/stress and other clinical factors in the modulation of innate inflammatory responses and immune-maturation in preterm infants in the neonatal intensive care unit (NICU). Fecal calprotectin (FCP), a non-invasive stable protein biomarker that was expressed primarily by neutrophils was used to measure intestinal inflammation.

The study found that the FCP level in preterm infants during the first four postnatal weeks were higher than healthy term infants and adults, and presented with a wide range of variation both inter- and intra-individually. The mean FCP value decreased significantly from the first week to the second week and then increased till the fourth week. These findings reflect the migration of neutrophil granulocytes and macrophages to the intestinal mucosa, may suggest the immaturity of the immune system in preterm infants' gut and the high permeability of gut mucosa.

Chronic (prolonged) pain/stress derived from daily medical procedures in the NICU is positively associated with FCP levels after controlling for the confounding factors, including mother's own milk, pre-rupture of membrane and antibiotics use during the first 72 hours. Results of this study provide, to the best of our knowledge, the first evidence for the modulation of chronic (prolonged) pain/stress in the early programming of the gut immune system in preterm infants. Further research needs to be conducted to enhance our understanding of the underlying mechanisms of chronic pain/stress in modulating the gut inflammatory process.

5.5 Limitations

The study presented in Chapter 2 intended to develop and test a tool to quantitatively measuring preterm infants' cumulative pain and stress. The final score was calculated based on the quantity/duration and the severity of each painful/stressful procedure that infants experienced in the NICU. One of the limitations of this instrument development study is the failure to

incorporate information related to the pharmacological and nonpharmacological interventions, which are important factors that affect the perceived pain level of each procedure. Furthermore, the study results do not account fully for variability in pain/stress experience across different neonates' conditions (i.e., gestational age) and different institutional contexts. These limitations potentially affect the sensitivity and specificity of the instrument and need to be considered in further development and validation of this instrument.

The study in chapter 3 is innovative in investigating the relationship between the systemic bacterial microbiome and plasma anti-CD4 autoantibodies in HIV+ subjects under long-term viral suppressive ART treatment. The findings showed exciting results that HIV-infected individuals with increased plasma anti-CD4 autoantibodies production presented a distinct microbiome pattern. However, due to the small samples size, the data failed to identify the bacterial species that were related to elevated autoantibodies production, which may be possible targets for intervention to suppress chronic auto-immune responses. In addition, the study did not analyze gut microbiome simultaneously with plasma microbiome. Although the data from the human gut microbiome project was used as a reference for the microbial composition comparison, the mechanisms of microbiome translocation from the gastrointestinal tract remains unclear.

The small sample size is the primary limitation of the study reported in Chapter 4. It has been suggested that a minimum of 30 subjects is an appropriate sample size in the mixed effects model (Raudenbush, Bryk, Bryk, & coaut, 2002). This study failed to meet the minimum level for detection of significance after accounting for missing data. Due to the large number of missing information related to the mother's antenatal conditions, such as medical complications and antibiotics use, the mother's information was not incorporated in the analysis. Also, further

investigation of other inflammatory markers will help to identify the stress-inflammation pathway and determine the sources and mechanisms of the inflammation.

5.6 Implication for practice, education and future research

Results of the present study provide new knowledge about modulation of immune responses by the brain-gut-microbiota axis. The knowledge gained allows us to better understand the interplay among stress, microbiota patterns and the activation of inflammation. These findings also expose further unresolved questions and indicate avenues for further research inquiry on the roles of brain-gut-microbiome in human health. Recommendations for clinical practices, education and future research will be considered below:

Cumulative pain/stress plays an important role in many aspects of infants' health, such as gut immune-hemostasis and neurobehavioral development. Development of Cumulative Pain/Stress Scales (APSS), a customized instrument offers considerable potential to track, measure and manage cumulative pain/stress in NICU setting. Awareness of cumulative pain/stress scores will also provide NICU nurses' the ability to identify the infants with an increased risk of adverse consequences, and to offer neuroprotective interventions, such as skin-to-skin contact, to promote more favorable developmental outcomes.

Results from Chapters 3 and 4 suggest that the enteral bacterial composition and function play an important role in modulating the immune responses. Thus, the involvement of gut microbiome and microbial translocation in mediating gut immune responses will be a necessary step. Incorporating gut microbiome patterns, specifically microbial diversity, composition, and function will help us further understanding of the roles of pain/stress and other external stimuli in the modulation of FC expression in preterm infants, and more generally, the immune responses. Understanding the role of gut microbiome will provide knowledge to develop the individualized

intervention that targeting on individual gut microbiome community, in order to modulate immune responses.

The study presented in Chapter 4 provides preliminary information for generating normative values of FCP in preterm infants. Results of the study revealed the effects of cumulative pain/stress, early enteral feeding, antibiotics use and pre-rupture of membranes on FCP levels in preterm infants during the first four postnatal weeks. The finding advanced current knowledge on the role of cumulative pain/stress in infants' health outcomes. More research is needed that explores how the gut inflammation mediate the impacts of cumulative pain/stress, and other clinical factors (i.e., feeding regimen, antibiotics use) on preterm infants' health outcomes, particularly neuro-behavioral development. Other inflammatory mediators, for example, pro-inflammatory cytokines and gut microbiome need to be analyzed simultaneously with FCP to map the inflammatory pathways and to identify the sources and consequence of the inflammation.

5.7 Conclusion

Emergent evidence suggested that brain-gut-microbiome axis plays an essential role in human health and disease. Results from studies within this dissertation advanced our knowledge in understanding the interplay of stress, microbiome and immune responses.

Preterm infants are born with the immature immune system. Cumulative pain/stress during the neonatal period, together with other factors such as feeding practice and antibiotics therapy can cause immune activation and production of calprotectin, thus altering immune function. Understanding of postnatal modification of the preterm immune system will allow development of individualized interventions to minimize unfavorable immune consequences.

Persistent systemic immune activation has been a critical issue and driving force of CD4+ T-cell loss even in HIV-infected patients under suppressive antiretroviral therapy. Results from this study revealed that plasma microbial composition in ART-treated HIV-infected subjects was associated with anti-CD4 autoantibodies. This finding suggested that gut and plasma microbiome may be an important target to reconstitute their peripheral CD4+ T cell counts. It provides a new avenue to develop a specific intervention to reconstitute their peripheral CD4+ T cell counts, reduce the risks of complications, morbidity and mortality in HIV patients.

To further understanding the mechanisms of the interplay of stress, microbiome and host immunity, a larger-scale study is needed to accommodate for additional factors, including gut microbial patterns (diversity and abundance), other immune factors such as cytokines profile, toll-like receptors and immune cells, anti-inflammatory agents such as cortisol. Future studies should focus on the exploration of the long-term effects of microbial dysbiosis and immune dysfunction, particularly in the preterm infants. Understanding the mechanism will help with the development of an individualized intervention to restore microbial symbiosis and immune function, reducing morbidity and mortality rates caused by immune dysregulation and ultimately improve human health.

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